University of Alberta

Induction of alcohol dehydrogenase, lactate dehydrogenase, and alanine aminotransferase gene expression in roots of *Arabidopsis thaliana* exposed to hypoxia

by

Kathleen Anne Sprysak

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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To Mom and Dad, who never quite knew what I was doing but who were always happy when things worked

Abstract

Alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and alanine aminotransferase (AlaAT) expression patterns were examined in roots of *Arabidopsis thaliana* exposed to hypoxic conditions. The amount of ADH activity increased 6 fold, relative to aerobic levels, after 96 h of hypoxia, whereas LDH and AlaAT each displayed a 2 fold induction of activity. Elevated amounts of the ADH, LDH, and AlaAT proteins were also detected following a 96 h hypoxic treatment. At the level of transcription, *Adh* mRNA accumulated to 30 times aerobic levels after 48 h of hypoxia. For *Ldh*, transcript levels increased 7 fold over 16 h. The *AlaAT* mRNA levels rose 10 fold over 24 h.

The *Arabidopsis AlaAT* gene was cloned and characterized. Sequence comparisons revealed homology with monocot *AlaAT*s. The induction of ADH, LDH, and AlaAT gene expression in roots suggests that these enzymes may be functionally important in the anaerobic stress response of *Arabidopsis thaliana*.

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List of Abbreviations

ABA = abscisic acid
ADH = alcohol dehydrogenase
ADP = adenosine diphosphate
AlaAT = alanine aminotransferase
ANP = anaerobic polypeptide
ARE = anaerobic responsive element
ARF = ARE (anaerobic responsive element) binding factor
AtHb1 = *Arabidopsis thaliana* haemoglobin 1
ATP = adenosine triphosphate

BCIP = 5-bromo-4-chloro-3-indolyl phosphate
bp = base pair
BSA = bovine serum albumin

C_3 units = compounds containing 3 carbon molecules
C_4 plants = plants that undergo C_4 photosynthesis
Cd = cadmium
cDNA = complementary DNA (deoxyribonucleic acid)
CH_4 = methane
cm^2 = square centimeters
CO_2 = carbon dioxide
cpm = counts per minute
Cu = copper

d = day(s)
dCTP = deoxycytosine triphosphate
DEPC = diethyl pyrocarbonate
DMS = dimethyl sulfate
DNA = deoxyribonucleic acid
dT = deoxythymidine
DTT = dithiothreitol
EC = enzyme classification
EDTA = ethylenediaminetetraacetic acid
EGTA = ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid

EMS = ethyl methanesulfonate

ER = endoplasmic reticulum

EST = expressed sequence tag

Exo III = exonuclease III

Fe = iron

Fe_2O_3 = ferric oxide

FW = fresh weight

g = gram(s)

G-box = sequence motif of 5'-CCACGT-3'

GABA = γ-aminobutyric acid

GBF1 = G-box binding factor 1

GCBP = GC binding factor

GSRE = general stress responsive element

GUS = β-glucuronidase

h = hour(s)

H^+ = hydrogen ion; proton

H_2O = water

HCl = hydrogen chloride

HPT = hypoxically pretreated

IgG = immunoglobulin G

in vitro = outside the organism

in vivo = in a living cell or organism

IU = international units

kb = kilobase(s)

kD = kilodalton

kPa = kilopascals

L = liter(s)

LDH = lactate dehydrogenase

M = molar

µg = microgram(s)

mg = milligram(s)

min = minute(s)

µl = microliter(s)

ml = milliliter(s)

mM = millimolar

µmol = micromole(s)

mmol = millimole(s)

Mn = manganese

$Mn(OH)_2$ = manganese II hydroxide

MND = magnitude not determined

MnO_2 = manganese IV oxide

MOPS = morpholinopropanesulfonic acid

mRNA = messenger RNA (ribonucleic acid)

N-limited = nitrogen-limited

N_2 = nitrogen gas

NAD^+ = nicotinamide adenine dinucleotide (oxidized form)

NADH = nicotinamide adenine dinucleotide (reduced form)

NADP = nicotinamide adenine dinucleotide phosphate

nm = nanometer(s)

NMR = nuclear magnetic resonance

NO_3^- = nitrate ion

O = oxygen

O_2 = oxygen gas

OD = optical density

PAGE = polyacrylamide gel electrophoresis

PBST = phosphate - buffered saline with Tween 20

PDC = pyruvate decarboxylase

pH = the -log of the concentration of hydrogen ions in a solution

P_i = inorganic phosphate

PLP = pyridoxal-5-phosphate

R001 = ADH^- mutant

S = sulfur
s = second(s)
SDS = sodium dodecylsulfate
SM buffer = sodium - magnesium buffer
SSC = sodium chloride and sodium citrate solution
SSPE = sodium chloride, sodium dihydrogen orthophosphate, and EDTA solution

T3 = T3 promoter (used as a primer for sequencing)
T7 = T7 promoter (used as a primer for sequencing)
TBST = Tris - buffered saline with Tween 20
TCA = tricarboxylic acid

V = volts
v/v = volume per volume

w/v = weight per volume
WT = wild-type

XET = xyloglucan *endo*transglycosylase

1 Introduction

Plant growth is noticeably impaired by the hypoxic (low oxygen) conditions imposed by flooding. A low oxygen environment can be created in many different ways. The most common means is the saturation of dryland soils by heavy rainfall. Soil oxygen deficiencies also are common in wetland areas that are regularly flooded, as well as in poorly-draining soils subjected to seasonal irrigation (Ponnamperuma 1972; Kozlowski 1984). When the oxygen supply becomes limited, plants cannot survive for extended periods of time. As a result, soil flooding poses a significant concern for agricultural crops. For example, the National Agricultural Statistics Service in the United States estimated that in 1993, approximately 20 million acres of corn and soybean crops suffered flood damage in the Mid-Western United States alone (Suszkiw 1994).

Plants vary widely in their abilities to withstand a low oxygen environment. Sensitive plant species like pea (*Pisum sativum*) and tobacco (*Nicotiana tabacum*) can survive hours of submersion, whereas rice (*Oryza sativa*) and barnyard grass (*Echinochloa phyllopogon*) can endure these conditions for days (Davies et al. 1974; Kramer 1951; Bertani et al. 1980). One type of adaptation found within many plants involves structural modifications, primarily within the roots. The efficiency of internal oxygen transport from the shoots to the roots may be increased to lessen the severity of the oxygen deficit (Woolley 1983). In addition to structural changes, plants can alter their metabolism and activate alternative fermentation pathways in roots to generate energy under conditions of oxygen stress (Davies et al. 1974). A plant's ability to survive an oxygen deficiency depends on the speed and effectiveness to which it adapts to its unfavorable environment.

Plant species with varying degrees of flooding tolerance have been studied to determine the nature of the anaerobic stress response. By examining both sensitive and tolerant varieties of plants, attempts can be made to determine how some plants endure low oxygen conditions more successfully than others. The actual mechanisms by which plant cells sense declining oxygen levels and communicate this message to neighboring cells or to other parts of the plant remain unknown. Individual cells must also internalize this signal, such that it reaches the nucleus where the stress-induced changes in gene expression patterns can occur. Much of the research in the area of anaerobic stress has focused on the oxygen deprived root environment and the anatomical and physiological alterations occurring within a plant. Recently, efforts have shifted towards attaining an understanding of the molecular changes taking place within plant

tissues exposed to oxygen stress, with the long term goal of defining the cascade of events responsible for bringing about the anaerobic stress response in plants.

In characterizing the responses of plants to flooding, discrepancies have arisen with respect to the terminology used to describe the amount of oxygen present in an "anaerobic" treatment. The term "hypoxia" commonly refers to a reduction in oxygen levels such that the rate of oxidative phosphorylation decreases and the production of ATP by this pathway is limited (Pradet and Bomsel 1978; He et al. 1996). "Anoxia" describes an environment in which the amount of oxygen present is so low that the synthesis of ATP by mitochondrial oxidative phosphorylation is negligible (Pradet and Bomsel 1978; He et al. 1996). Under "anoxic" conditions, glycolysis and fermentation are primarily responsible for ATP production. The word "anaerobic" refers to oxygen-free conditions, although this term has been used in the literature to describe a more general reduction of oxygen (Andrews et al. 1993). An accurate account of the degree of oxygen deprivation is essential in describing the observed "anaerobic response", as the magnitude of a plant's response to flooding conditions noticeably varies with the severity of the stress treatment. For example, plant roots are more tolerant of anoxic conditions if the level of oxygen is decreased gradually (hypoxic acclimation), as opposed to the immediate exposure to an oxygen-free environment (Saglio et al. 1988; Cobb et al. 1995). These three terms have been used in this thesis as outlined above by Pradet and Bomsel (1978); however, in differentiating plant responses to varying levels of oxygen, specific references to the appropriate term will be provided.

1.1 Soil Dynamics under Flooding Conditions

In drained, well-structured soil, oxygen levels closely resemble those in ambient air (Russell 1973). The presence of large air-filled pores and cracks in the soil facilitate rapid diffusion of gases between the atmosphere and soil. Under flooding conditions, excess water essentially eliminates these gas-filled pores, reducing gas exchange between the soil and atmosphere by approximately 10,000 fold (Greenwood 1961). Molecular oxygen in the soil is consumed by the respiration of roots and soil microorganisms within a few hours (Drew and Lynch 1980). Much of the previously aerated soil becomes oxygen deficient, with the exception of an oxygenated transition zone ranging from a few millimeters to a few centimeters below the soil surface (Patrick and DeLaune 1972; Ponnamperuma 1984). The oxygen deficit beneath the soil surface alters the oxidation-reduction status of the soil. The limited oxygen conditions

imposed by flooding require respiring soil organisms to use electron acceptors other than oxygen.

1.1.1 Alternative terminal electron acceptors

A variety of alternative oxidants are present in the soil. These oxidants are utilized in a specific order. Following oxygen depletion from the soil, nitrate (NO_3^-) ions are reduced to nitrogen gas (N_2) or ammonium (NH_4^+) ions within a few days (Ponnamperuma 1972). Reduction of manganese IV oxide (MnO_2) ensues, resulting in the production of manganese cations (Mn^{2+}), which are more readily taken up by the plant's root system (Ponnamperuma 1972). Elevated levels of Mn^{2+} ions are phytotoxic and are associated with aerial tissue damage of flooded plants (Drew and Lynch 1980). High radial oxygen diffusion at the root surface promotes the precipitation of manganese II hydroxide ($Mn[OH]_2$), along with other toxic ions (Ando et al. 1983). After nitrate and manganese IV oxide are consumed, the reduction of ferric oxide (Fe_2O_3) begins (Ponnamperuma 1972). Ferrous (Fe^{2+}) ions can accumulate to millimolar concentrations in waterlogged soils, and substantial increases in iron levels have been reported in plants growing in inundated soils (Ponnamperuma 1972; 1984). The locally oxidized atmosphere in and around plant roots results in the formation of reddish-brown plaques of ferrous hydroxide ($Fe[OH]_2$), which reduce the uptake of Fe^{2+}, along with heavy metals such as zinc (Zn^{2+}), cadmium (Cd^{2+}), copper (Cu^{2+}), and manganese (Mn^{2+}) ions that bind to ferrous hydroxide (Justin and Armstrong 1987; Otte et al. 1989). Following the reduction of ferric oxide, soil microbes start converting sulfate (SO_4^{2-}) ions to sulfide (S^{2-}) ions (Ponnamperuma 1972). Sulfide ions freely enter the plant and have been associated with the inhibition of cytochrome oxidase activity, resulting in a blockage of aerobic respiration (Ernst 1990). Sulfides also are highly reactive and form insoluble metal sulfides with toxic heavy metal ions, including Zn^{2+}, Cu^{2+}, Cd^{2+}, and Fe^{2+} (Engler and Patrick 1975). Carbon dioxide, the final oxidant in waterlogged soils, is reduced to methane gas (CH_4) by microorganisms (Ponnamperuma 1972).

1.1.2 Phytotoxins produced by soil microbes

In addition to altering the redox potential of the inundated soils, microorganisms produce harmful organic acids during the anaerobic decomposition of plant residues. Acetic, propionic, and butyric acid are among the organic acids accumulating under flooding conditions, with acetic acid being produced in the highest amount (Drew and

Lynch 1980). Organic acids increase membrane permeability in the cells of the roots (Lee 1977). Secondary effects of phytotoxic organic acid levels include decreased root elongation, reduced initiation of new roots, and impaired uptake of potassium and phosphate ions (Ponnamperuma 1984).

1.2 Flood-Induced Changes in Plant Anatomy and Physiology

Flooding conditions induce many structural and physiological modifications occurring within the plant. As the soil becomes inundated with water, roots are the first plant tissue faced with the problem of an oxygen deficiency. Characteristic symptoms of flood damage also appear in the shoots. Visible changes in plant morphology are indicative of the plant's attempts to adapt to growth in a waterlogged soil.

1.2.1 Roots in a low oxygen environment

Even before dissolved soil oxygen is totally depleted, patterns of root growth are altered. The rate of root extension slows down and eventually ceases as a result of an insufficient oxygen supply (Drew 1992; Huck 1970). The root apical meristem is particularly sensitive to oxygen levels, because of its high oxygen demand. In this region, cell division and elongation are halted (Huck 1970).

A low oxygen environment also induces changes in the structural organization of roots. Many cereal crops including wheat, barley, and maize develop narrower, shorter roots of greater porosity under oxygen limitation (Armstrong 1979). The growth of lateral roots in the surface layer of the soil is stimulated, and adventitious roots emerge from the base of the stem and grow into the top layer of the soil (Glinski and Stepniewski 1985; Kramer 1951; Jackson 1955). Over the short term, these shallow roots help to supplement the transport of water and nutrients to the shoots (Kramer 1951; Jackson 1955). Changes also can occur in the direction of root growth. Some plants avoid the reduced oxygen conditions below the soil surface by stimulating upward growth of roots towards the more oxygenated surface layer of the soil (Armstrong and Boatman 1967).

Soil waterlogging not only interferes with the structure and organization of the root system but disrupts root function as well. Flooding causes a rapid decrease in the ability of roots to absorb and conduct water, even from saturated soils (Kramer 1951). Radial ion transport by the roots to the xylem is also inhibited under oxygen deficient conditions (Drew 1990). These problems with root function may be attributed to limited functioning of the conducting machinery. Water conduction by the roots may be partially blocked by the bacterial decomposition of dying roots (Kramer 1951). The

remaining viable root tissues have relatively low energy levels, compared to their aerobically growing counterparts, which negatively affect the energy-dependent process of ion transport (Drew 1992).

1.2.2 Effects of flooding on shoots

Although the root system is directly affected by oxygen limitation in waterlogged soils, flood damage also can be found in the aerial portions of the plant. The rate of leaf and shoot extension decreases, resulting in a reduction of the leaf mass and yield (Drew and Lynch 1980). Leaves and shoots also begin to wilt, primarily because of decreased water uptake by the roots (Drew and Lynch 1980). Older leaves become chlorotic and senesce prematurely, a symptom which correlates with the redistribution of phloem-mobile nutrients (nitrate, phosphate, and potassium ions) to younger leaves (Drew and Lynch 1980; Trought and Drew 1980).

The effects of reduced water uptake by the root system also are associated with stomatal closure in the aerial tissues (Sojka et al. 1975). Stomatal closure helps to minimize transpirational water loss but also results in decreased rates of photosynthesis (Sojka et al. 1975). Stomatal closure has been linked with elevated levels of the plant growth regulator abscisic acid and also with reduced potassium ion concentrations in the leaves of flooded plants (Peaslee and Moss 1966). Regardless of the triggering signal, stomata may remain closed even after the oxygen stress has been alleviated (Sojka 1992).

Another hormonally-induced shoot response to a reduced oxygen environment is leaf epinasty. The plant growth regulator ethylene promotes leaf epinasty within the first few days of flooding (Bradford and Yang 1980). Epinasty results from an enhancement of cell expansion on the upper (adaxial) surface of the leaf petiole, causing a downward growth of the leaf (Drew 1990; Armstrong et al. 1994). This adaptive response by the plant may reduce the interception of sunlight by leaves and limit water loss due to transpiration (Drew and Lynch 1980).

1.2.3 Structural changes within root tissue

Plant shoots can act as a source of oxygen for the roots. Internal transport of oxygen from the aerial parts of the plant to the roots occurs via an interconnected network of gas-filled spaces (lacunae) which make up aerenchyma (Armstrong 1979). These air-filled pores may form in one of two ways. Schizogenous aerenchyma formation occurs by cell separation, resulting from modified patterns of cell division within the

root meristem or changes in the intercellular extensibility of cells near the meristem (Justin and Armstrong 1987; Armstrong et al. 1994). This type of aerenchyma forms in many flood-tolerant wetland species like *Senecio congestus* and *Rumex maritimus* (Bakker 1960; Laan et al. 1989). Lysigenous aerenchyma develops as a result of the lysis of cells within the mid-cortex of the root. Lysigenous aerenchyma formation is seen in rice (*Oryza sativa*) and maize (*Zea mays*) (Figure 1.1; Drew and Stolzy 1991; Konings and Lambers 1990). In many wetland species, aerenchyma formation occurs constitutively, whereas in dryland species like barley, wheat, and maize, lysigenous aerenchyma development is an adaptive response to oxygen stress (Webb and Jackson 1986; Campbell and Drew 1983; Jackson and Drew 1984). Elevated levels of endogenous ethylene in the roots and increased amounts of soil ethylene appear to induce aerenchyma formation in dryland plant species (Drew et al. 1979). Formation of lysigenous aerenchyma also appears to be dependent on levels of intracellular calcium. He et al. (1996) suggested that the cellular processing of the ethylene signal brings about an increase in intracellular calcium, which activates a series of unknown events ultimately resulting in cell degeneration.

Aerenchyma formation has many survival benefits for dryland plants faced with the problem of flooding. Large gas-filled spaces decrease the volume of oxygen-consuming tissue in the roots, as fewer respiring cells are present (Drew and Lynch 1980). Aerenchyma production also increases the efficiency of internal oxygen transport from the shoots to the submerged root system by reducing the physical resistance to diffusion (Justin and Armstrong 1987). Elevated rates of internal oxygen diffusion result in higher energy levels in the roots compared with their non-aerenchymatous counterparts under oxygen stress (Drew et al. 1985). Although oxygen transport through aerenchyma may not restore aerobic respiration in root cells, the improved internal aeration will help the plant to maintain a functional root system (Drew and Stolzy 1991).

The structural advantages of aerenchyma formation have been extensively studied; however, little is known about its development at the level of gene expression. Recently, a gene (*1005*) has been identified in maize that may play a role in aerenchyma development (Peschke and Sachs 1994). The protein product of gene *1005* shows a high degree of similarity to the xyloglucan *endo*transglycosylase (XET) family of proteins (Saab and Sachs 1995). XET cDNAs from a variety of other species, including *Arabidopsis thaliana*, tomato (*Lycopersicon esculentum*), and wheat (*Triticum aestivum*) have been isolated, and high stretches of homology have been found between the predicted protein sequences (Okazawa et al. 1993). The proposed function of the XET

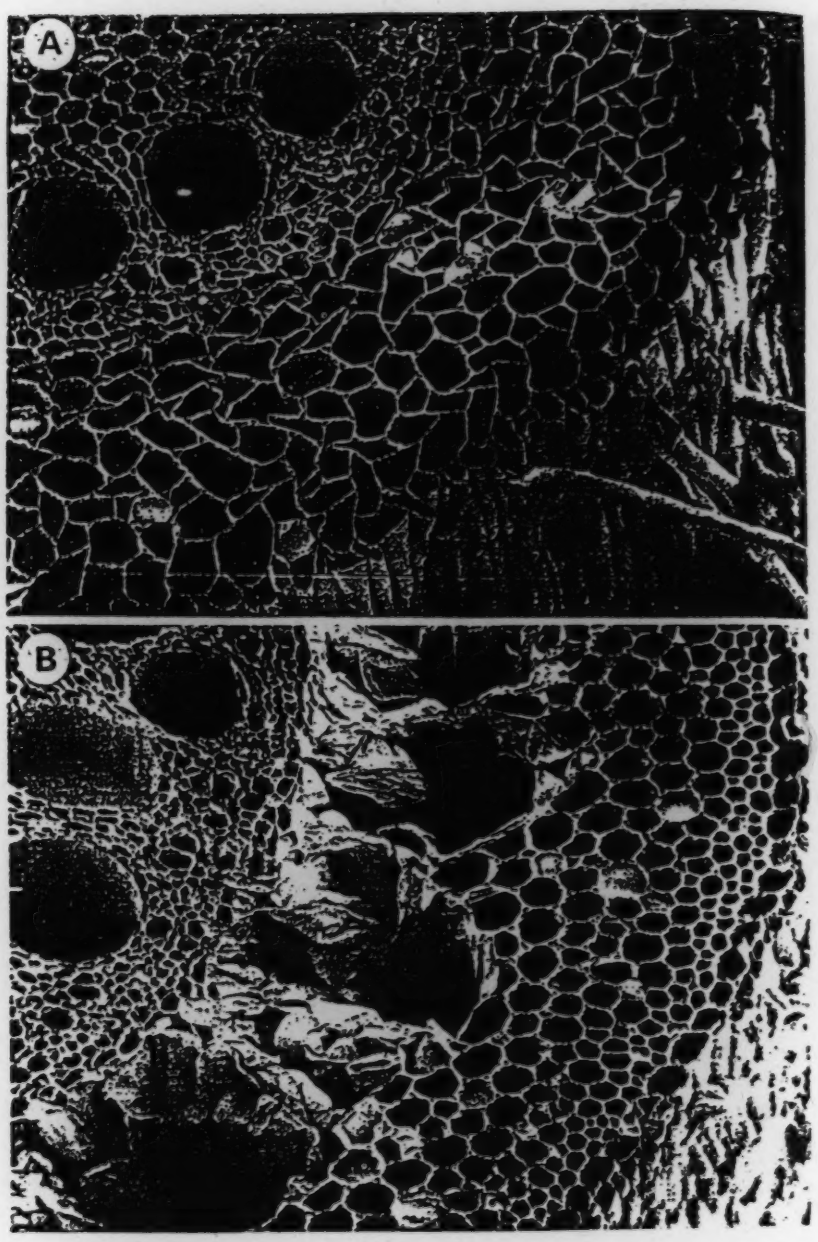

Figure 1.1. Scanning electron micrograph of lysigenous aerenchyma in root tissues of 3 to 4 day-old maize seedlings. A. A section from a well-aerated root. B. A section from a root grown in a low oxygen environment (3 to 12.5 kPa of oxygen). Aerenchyma formation is occurring by cell lysis in the cortex that lies between the epidermis (bottom right) and the stile (top left). From Jackson (1990), p. 54

protein involves the manipulation of cell wall construction during seed germination, cell enlargement, and fruit ripening by acting on xyloglucan polymers within the matrix of the cell wall (Fry et al. 1992; Redgwell and Fry 1993; Sachs et al. 1996). Xyloglucans consist of β-1, 4 linked glucans with xylosyl side chains (Fry 1989). Because xyloglucans can undergo tight hydrogen bonding with cellulose, they may function as tethers, cross-linking adjacent cellulose microfibrils for added cell wall strength (Xu et al. 1995; Sachs et al. 1996). XET enzymes modify cell wall structure by the internal cutting and rejoining of xyloglucan polymers to allow for an increase in cell volume while maintaining cell wall integrity.

Aerenchyma formation in oxygen-deprived roots is also associated with elevated levels of cellulase and other cell wall degrading enzymes, along with increased amounts of the plant growth regulator ethylene (Drew et al. 1979; He et al. 1996). The potential role of XET in the development of aerenchyma has been strengthened by its pattern of expression. Maize XET mRNA levels increased in root tissue upon exposure to low oxygen conditions, peaking after 12 h (Saab and Sachs 1996). Aerenchyma formation was observed 48 h after hypoxic induction (Saab and Sachs 1996). To determine the responsiveness of *1005* to ethylene, XET transcription was monitored following treatment with (aminooxy)acetic acid, an ethylene inhibitor, under flooding conditions. Roots treated with the inhibitor displayed significantly reduced levels of transcript accumulation (Saab and Sachs 1996). Furthermore, the amount *1005* mRNA increased when aerobic maize seedlings were exposed to high levels of exogenous ethylene (Saab and Sachs 1996). The proposed function of XET as a cell wall loosening enzyme, along with its transcriptional induction pattern, support the possible role of xyloglucan *endo*transglycosylase in the development of aerenchyma in maize roots under flooding conditions.

In summary, plants respond to a low oxygen environment with a variety of structural and physiological changes to increase their chances of survival. Formation of aerenchyma improves oxygen transport to the roots. Shoots also modify their pattern of growth and development to compensate for root dysfunction. Both the anatomical and physiological adaptations of a plant play an important role in surviving oxygen stress.

1.3 Effects of Oxygen Deficiency at the Ultrastructural Level

1.3.1 Plant mitochondria

Low oxygen stress brings about many changes in plant structure and function at the cellular level. The mitochondria within plant cells are of particular interest because

of their involvement in aerobic metabolism. An example of mitochondrial development under anoxia occurs in the rice coleoptile. When germination occurs in an oxygen-free environment, mitochondria develop normally for the first 48 h (Opik 1973). The mitochondria have an oval shape and contain many elongated cristae that are randomly arranged within the organelle (Vartapetian et al. 1976; Andreev et al. 1991). Two days after seed germination, swollen mitochondria are observed under anoxic conditions (Vartapetian et al. 1978). This enlargement of the mitochondria also has been observed in submerged maize seedlings (Aldrich et al. 1985). Further examination of the rice coleoptile, along with intact seedlings of rice and tomato, revealed a reduction in the number of cristae; these cristae were arranged in parallel stacks within the mitochondria (Figure 1.2) (Vartapetian et al. 1976; 1978). In addition to these structural abnormalities, the mitochondrial matrix appeared transparent in the rice coleoptiles and seedlings, as well as in maize seedlings (Vartapetian et al. 1976; Aldrich et al. 1985). Prolonged exposure to anoxic conditions resulted in complete mitochondrial degeneration in rice, maize, and wheat seedlings (Vartapetian et al. 1976; 1985).

Despite this apparently severe damage, re-exposure to aerobic conditions can reverse the degradation process (Vartapetian et al. 1985). Symptoms of mitochondrial damage occurred more rapidly in excised roots, leaves, and coleoptiles than in intact seedlings (Vartapetian et al. 1976). Partial to complete recovery of mitochondria in excised organs was observed when 0.5% to 3% glucose was added to the submersion buffer (Vartapetian et al. 1976; Vartapetian and Poljakova 1994). Exogenous glucose extended tissue survival to that of intact seedlings (Vartapetian et al. 1978).

The results of these glucose supplementation experiments indicate that the availability of carbon plays an important role in surviving anoxia. Excised plant organs may not have access to stored sugars or other substrates which may be required to maintain sufficient energy production (Vartapetian et al. 1978). Vartapetian and colleagues (1978) suggested that the primary cause of mitochondrial degeneration was carbon starvation, as opposed to tissue asphyxiation or the toxicity of anaerobic metabolites. The availability of carbon sources, however, cannot be the only factor involved in the survival of anoxia. The extended tolerance of excised tissues supplied with exogenous glucose was limited, suggesting the involvement of other factors in anoxia survival (Vartapetian and Poljakova 1994).

Figure 1.2. Electron micrographs of mitochondria in the roots of rice (*Oryza sativa*). A. Aerobic root cortex cells containing normal mitochondria with randomly arranged cristae. B. Root cortex cells after 2 d of anoxia, following aerobic germination, with enlarged mitochondria, containing reduced numbers of cristae in a parallel arrangement. Abbreviations: ER, endoplasmic reticulum; M, mitochondria. From Vartapetian et al. (1976), p. 221.

1.3.2 Other ultrastructural changes

Exposure to anoxic conditions affects the development of other organelles and structures within the cell. Electron microscope photographs of the endoplasmic reticulum (ER) revealed a reduction in the number of ribosomes present in rice coleoptiles after 6 d of anoxia (Vartapetian et al. 1978). The majority of ribosomes existed as single entities in the cytoplasm of anoxic rice coleoptiles, as well as in submerged soybean and maize roots. Under aerobic conditions, ribosomes aggregated into polysomes for protein translation (Rascio et al. 1987; Lin and Key 1967; Bailey-Serres and Freeling 1990). Plastids within the cells of rice coleoptiles became wrinkled after 96 h in an oxygen-free environment (Ueda and Tsuji 1971). The altered appearances of the plastids and ER may be indicative of the inability of energy deficient cells to preserve organelle integrity (Ueda and Tsuji 1971).

At the DNA level, studies in submerged maize seedlings revealed the presence of highly condensed chromatin in the nuclei. This chromatin later became partially dispersed (Aldrich et al. 1985). Chromatin compaction has been associated with reduced levels of gene transcription, and an overall decrease in transcription correlates well with the small group of anaerobic proteins expressed under anoxic conditions. This provides a nice link between the structural and biochemical data (Sachs et al. 1980).

1.4 Plant Metabolism under Flooding Conditions

Structural adaptations in response to oxygen deficient conditions are accompanied by an active rearrangement of cellular metabolism. Oxidative respiration is greatly reduced in roots growing in waterlogged soils, as little oxygen is available to act as the terminal electron acceptor in the electron transport chain. Glycolysis can continue, provided NAD^+ regeneration occurs, but the breakdown of pyruvate, the end product of glycolysis, by the tricarboxylic acid (TCA) cycle is reduced under low oxygen conditions. Instead, pyruvate is shunted into alternative fermentation pathways that can proceed in the absence of oxygen, but with a decrease in net ATP production.

The amount of energy generated by aerobic respiration compared to anaerobic glycolysis differs significantly. The complete breakdown of glucose to carbon dioxide and water by glycolysis and the TCA cycle yields 38 moles of ATP. The glycolytic pathway yields only 2 ATP per molecule of glucose. In a reduced oxygen environment, pyruvate commonly is converted to ethanol or lactate, resulting in the oxidation of NADH to NAD^+. The fermentative pathways adopted by many plant species under conditions of oxygen stress are highly inefficient in energy production compared to aerobic respiration. To

compensate for reduced ATP synthesis, the rate of glycolysis may be accelerated, resulting in a Pasteur effect (Crawford 1967; Glinski and Stepniewski 1985). However, root cells have a high rate of energy turnover, particularly in the root tips. The reduction of ATP production within these cells under flooding conditions manifests itself in decreased rates of protein and nucleic acid synthesis, along with lowered rates of other ATP-consuming processes (Drew 1990). Andreev and colleagues (1991) proposed that the ability of a plant to survive oxygen deficient conditions depends largely on the energy status of its cells.

1.4.1 Metabolism and flooding tolerance: Crawford's theory

In his theory of flooding tolerance, Crawford hypothesized that a plant's ability to withstand low oxygen conditions was inversely related to its capacity for ethanol production (McManmon and Crawford 1971). This theory was based on the flooding responses of six species previously classified as flood tolerant or flood sensitive. Intolerant plants responded to oxygen deprivation by accelerating the glycolytic breakdown of sugars to pyruvate, which subsequently was converted to acetaldehyde and then to ethanol. According to this theory, elevated levels of ethanol in these plants would contribute to the poisoning of cellular metabolism. Plants able to survive flooding conditions did not increase the rate of glycolysis nor did they induce alcohol dehydrogenase (ADH) activity for ethanol production. Instead of accumulating toxic levels of ethanol, tolerant species would synthesize malate in a two step reaction from phosphoenolpyruvate (Figure 1.3). Malate, a non-toxic metabolite, accumulated in the oxygen-stressed cells by the conversion of phosphoenolpyruvate to malate with the oxidation of NADH. Malic enzyme, which catalyzes conversion of malate to pyruvate, was not synthesized under these conditions. According to McManmon and Crawford (1971), elevated levels of ADH activity and ethanol production represented a high sensitivity to flooding, whereas high malate concentrations were indicative of flooding tolerance.

Since its proposal in 1971, many reports have discredited this initial theory of flooding tolerance. Contrary to Crawford's theory, the induction of ADH activity and subsequent ethanol fermentation have an important function in a plant's survival of an oxygen deficient environment. Rice, a flood tolerant plant, displays marked increases in ADH activity levels, along with a high rate of glycolysis to meet its ATP requirements. (Bertani et al. 1980; Davies 1980). Further studies in maize, a flood sensitive species, indicated an essential role for ADH in anaerobic tolerance. Maize ADH null mutants

succumb to oxygen stress within a few hours, whereas normal ADH^+ seedlings survive such conditions for one to three days (Schwartz 1969).

The correlation between ethanol accumulation and plant injury made by McManmon and Crawford (1971) has not been substantiated. The amounts of ethanol detected in submerged plant tissues are significantly lower than experimentally determined levels found to cause cell damage (Jackson et al. 1982; Alpi et al. 1985). Although plants can generally accommodate higher concentrations of ethanol than those found in nature, safeguarding mechanisms exist to limit intracellular ethanol accumulation. Rice, maize, barley (*Hordeum vulgare*), and sunflower (*Helianthus annuus*) roots exude excess ethanol into the external medium (Bertani et al. 1980; Davies 1980; Good and Muench 1993; Jayasekera et al. 1990). Surplus ethanol can also be transported to the shoots through the transpirational stream for subsequent metabolism or release into the air, as seen in pea, sunflower, and tomato plants (Jackson et al. 1982; Jayasekera et al. 1990; Fulton and Erickson 1964). Overall, the activation of ethanol fermentation in an oxygen limited environment appears more likely to benefit plants, as opposed to exerting a toxic effect.

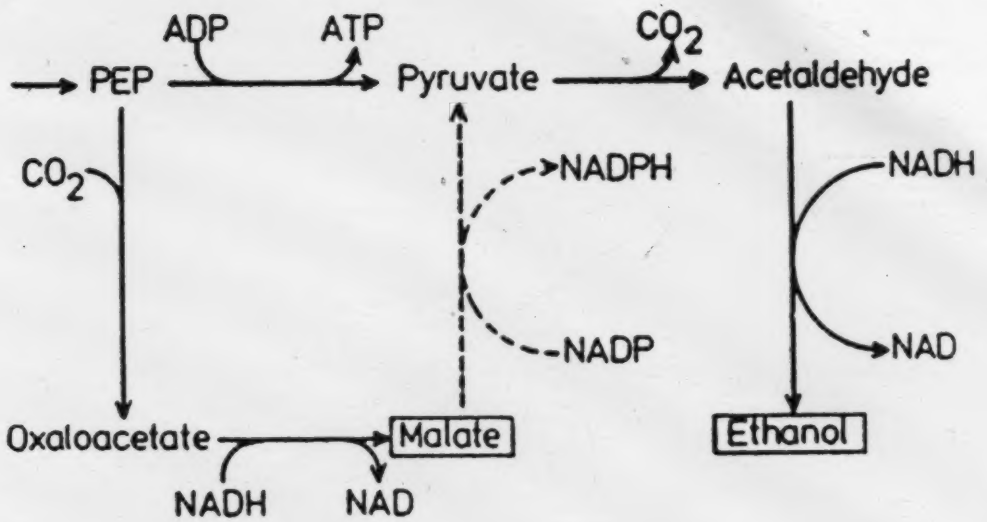

Figure 1.3. The proposed pathway for the production of malate (in flood tolerant species) and ethanol (in flood sensitive species), according to Crawford's theory. PEP, phosphoenolpyruvate. From Davies (1980), p. 585.

Scientists have also challenged the adaptive significance of malate synthesis in flood tolerance. The two step conversion of phosphoenolpyruvate to malate provides no net yield of ATP. Thus, anaerobic induction of this respiratory pathway would not benefit a plant faced with acute energy starvation (Jackson and Drew 1984). Malate production may be utilized by plant cells unable to tolerate high levels of pyruvate. This response, however, varies significantly between plant species (Joly 1994). Further, the detection of malic enzyme, which catalyzes the conversion of malate to pyruvate, in the roots of all the flood tolerant species used in Crawford's study (*Senecio aquaticus* L., *Caltha palustris* L., *Mentha aquatica* L., and *Ranunculus flammula* L.), has cast further doubts on the accuracy of this theory of flood tolerance.

1.4.2 Cytoplasmic acidosis and the pH-stat; the Davies-Roberts hypothesis

In the midst of the controversy surrounding Crawford's theory, another hypothesis emerged to explain how short term flooding tolerance may be attained. Davies and associates (1974) formulated a theory based on their experiments with pea seed extracts, which contained all the enzymes required for glycolytic and fermentative metabolism. When provided with the appropriate cofactors, these extracts could carry out the breakdown of glucose. Using this cell extract system, Davies et al. (1974) were able to follow the anaerobic progression of glycolysis and subsequent breakdown of pyruvate by measuring metabolite and pH levels at various time points. Results of these experiments indicated that lactate fermentation preceded ethanol production. Over time, pH levels in the reaction mixture decreased, accompanied by an increase in the amount of ethanol produced.

Davies and colleagues (1974) proposed that a plant initially responds to an oxygen deficit by activating lactate dehydrogenase (LDH), which catalyzes the production of lactate from pyruvate. This would provide a means of regenerating NAD^+ for glycolysis. At the onset of anaerobiosis, the cytoplasmic pH of a root cell is nearly neutral (pH 7.3 to 7.4). Activation of LDH is plausible under these conditions, as this enzyme works most efficiently at a slightly alkaline pH. With the induction of LDH, lactate begins to accumulate within the cell, and the cytoplasm becomes more acidic. This lower pH activates pyruvate decarboxylase (PDC), an enzyme with an acidic pH optimum, that catalyzes the first step in ethanolic fermentation (Figure 1.4). Conversion of pyruvate to acetaldehyde by PDC, and subsequently to ethanol by ADH, also results in the oxidation of NADH for continued glycolysis. The pH change has a negative effect on LDH activity, which declines with the falling pH levels. Thus, lactate fermentation ceases, whereas ethanol production increases and later plateaus. Davies and

associates (1974) postulated a role for these two enzymes as a type of metabolic pH-stat to regulate cytoplasmic pH in an oxygen limited environment.

Roberts and colleagues (1984a,b) strongly supported Davies' theory, suggesting that the switch from lactate to ethanol fermentation and the subsequent stabilization of pH could contribute to the increased survival of plant tissues relative to vertebrate tissues under hypoxic conditions. Roberts' group demonstrated the importance of cytoplasmic pH regulation in hypoxically stressed plant tissues. Thus, this theory is now referred to as the Davies-Roberts hypothesis. In studies with excised maize root tips, Roberts and coworkers (1982; 1984b) found that the cytoplasmic pH fell from 7.4 to 6.8 within 20 min of exposure to a reduced oxygen environment. Root tip cells could maintain this lower pH level for approximately 10 h (Roberts et al. 1984b). However, similar experiments with maize plants homozygous for the *Adh1* null mutation revealed no stabilization of pH (Roberts et al. 1984b). Cytoplasmic pH continued to drop in the root tips of *Adh1*$^-$ seedlings, probably due to a low rate of lactate fermentation (Roberts et al. 1984a,b). Roberts et al. (1984a) postulated that the ability of wild-type maize plants to survive longer periods of hypoxia than *Adh1*$^-$ seedlings was contingent on the delay of cytoplasmic acidosis.

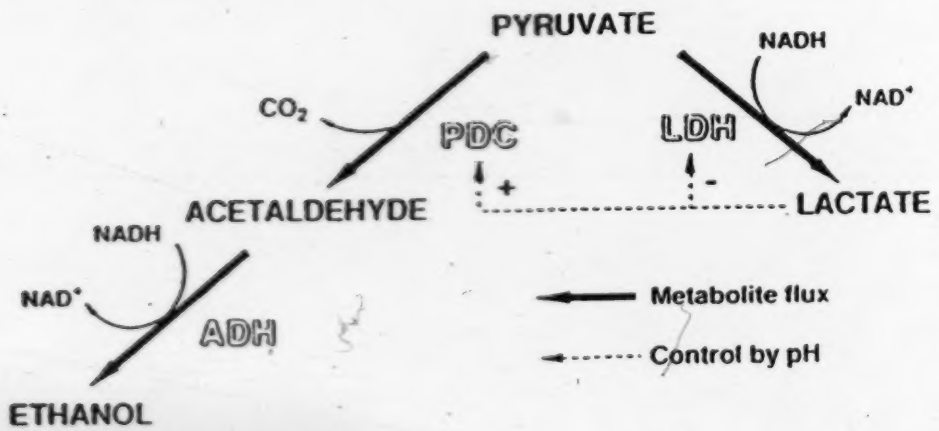

Figure 1.4. A schematic representation of the effect of lactate production on LDH and PDC, as described in the Davies-Roberts hypothesis. ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; LDH, lactate dehydrogenase. From Rivoal and Hanson (1994), p. 1180.

The operation of a metabolic pH-stat was further supported by experiments that manipulated either cytoplasmic pH or levels of gene expression. According to the Davies-Roberts hypothesis, cytoplasmic pH plays a critical role in the switch from lactate to ethanol production. Fox and colleagues (1995) examined the role of cytoplasmic pH as a signal for ethanol production. Previous studies had indicated that the 10 min lag between the onset of hypoxia and the accumulation of ethanol could be shortened by adding acetic acid to artificially lower the pH (Roberts et al. 1984b). Fox's group (1995) demonstrated the reversibility of this switch. In anoxic maize root tips, ethanolic fermentation was halted when the cytoplasmic pH was increased by the addition of methylamine (a weak base) to the external medium, following the initial drop in pH observed with the onset of anoxia (Fox et al. 1995). These findings provided strong evidence in favor of the Davies-Roberts theory, reinforcing the role of cytoplasmic pH regulation in flooding tolerance.

Levels of gene expression also have been altered to determine the effect on the flooding response. An LDH cDNA from barley was constitutively expressed in roots of tomato (Rivoal and Hanson 1994). Similar patterns of early lactate production and subsequent increases in the amounts of ethanol were observed in both transgenic and control roots under anoxic conditions (Rivoal and Hanson 1994). Despite the constitutive overexpression of the barley LDH cDNA, lactate production in tomato roots noticeably decreased within first hour of anoxia, in agreement with the Davies-Roberts hypothesis (Rivoal and Hanson 1994).

While the metabolic pH stat appears to play an adaptive role in flooding tolerance, many plant tissues eventually succumb to cytoplasmic acidosis. Correlations between a plant cell's ability to delay acidification of its cytoplasm and its ability to survive flooding conditions have been reported. For example, root tips of blackeye peas and navy beans tolerate a reduced oxygen environment for a relatively short period of time (9 h) compared with other flood sensitive species like pea (15 h), soybean (18 h), and maize (25 h) (Roberts et al. 1985). Examination of cytoplasmic pH levels during the first 16 h of hypoxia revealed a steady decline in these species with no stabilization (Roberts et al. 1985). Although these results provide good evidence for an association between cytoplasmic acidosis and cell death, the actual mechanism by which death occurs remains unknown. Acidification of the cytoplasm may also be promoted by vacuolar proton leakage and H^+ ion release in ATP hydrolysis. Reduced energy levels associated with hypoxia could also become insufficient to fuel the tonoplast H^+ - ATPase, which catalyzes the active transport of H^+ from the cytoplasm into the vacuole (pH 5.6) (Roberts et al. 1984a). In addition to tonoplast proton transport, the plasma membrane

H^+ - ATPase is stimulated under anoxic conditions (Xia and Roberts 1996). This proton pump exports H^+ ions from the cytoplasm to the cell exterior and increases in activity as the cytoplasmic pH falls, perhaps as a last resort to reduce the magnitude of the drop in pH (Xia and Roberts 1996). In spite of these attempts to learn more about cytoplasmic acidosis, the mechanism by which acidification brings about destruction of cells in the root meristem has yet to be elucidated. Researchers have speculated that cytoplasmic acidosis triggers a series of the harmful events, but an understanding of the precise mechanism resulting in cell death has yet to be attained (Roberts et al. 1984a; Kennedy et al. 1992; Xia and Roberts 1996).

Much support has been generated for the Davies-Roberts hypothesis in the last 20 years, but this theory is not without its shortcomings. To begin with, the universality of this hypothesis has been questioned. For the most part, flood sensitive plants like maize and pea show a similar pattern of a drop in cytoplasmic pH, followed by a stabilization period and ultimately another decline in pH. In some tolerant species like *Echinochloa phyllopogon* (barnyard grass) and rice, an alkalinization of the cytoplasm and vacuole occurs shortly after the onset of anoxia (Menegus et al. 1989; 1991). Problems with the Davies-Roberts theory also have arisen in relation to flood sensitive plants. Saint-Ges and coworkers (1991) found that the time course of lactate accumulation in excised maize root tips did not follow the pattern of cytoplasmic pH changes. For example, under hypoxic conditions, the cytoplasmic pH had dropped well before intracellular levels of lactate had plateaued, indicating that the falling pH does not result from lactate production alone (Saint-Ges et al. 1991). Studies in barley and some species of the halophytic genus *Limonium* also suggest that LDH activity and lactate production may have a more significant function during prolonged hypoxic exposure, as opposed to a transitionary role at the onset of hypoxia or anoxia (Hoffman et al. 1986; Rivoal and Hanson 1993). In addition, speculations regarding the existence of a lactate transporter, residing in the plasma membrane, have been put forward to account for the presence of lactate in the external medium (Rivoal and Hanson 1993; 1994). The accumulation of lactate within the cell is an essential component of the Davies-Roberts hypothesis; the postulated metabolic pH-stat would not work without it. Although the Davies-Roberts theory does have some shortcomings, it has helped to describe some of the major factors involved in short term flood tolerance.

1.4.3 Other metabolic strategies for flooding tolerance

Although neither the Davies-Roberts nor Crawford hypotheses can account for all of the metabolic responses occurring in tolerant and sensitive plants, both theories recognize the dramatic biochemical changes occurring within cells in a reduced oxygen environment. Many different plant species significantly increase their rate of ethanol fermentation, suggesting that the production of ethanol is universal under flooding conditions. In addition to the accumulation of ethanol, other metabolites have been found at elevated concentrations in various parts of a plant exposed to anoxia.

Succinate levels increase in some plant tissues in response to low oxygen stress. Along with its production in the submerged coleoptiles of rice, succinate also accumulates to varying degrees in the shoots of barnyard grass and in the N-limited (nitrogen-limited) green alga *Selenastrum minutum* (Menegus et al. 1988; 1989; Vanlerberghe et al. 1989). A less pronounced accumulation of succinate has been detected in the leaves of wheat and maize seedlings; however, no increase in succinate synthesis has been found in root tissues of rice or maize (Menegus et al. 1989; Fan et al. 1988; Roberts et al. 1992). One proposed pathway of succinate production involves partial operation of the tricarboxylic acid cycle, in which phosphoenolpyruvate is converted to oxaloacetate by phosphoenolpyruvate carboxylase. Oxaloacetate then enters the reductive pathway in which it is converted to malate, fumarate, and subsequently succinate (Figure 1.5) (Vanlerberghe et al. 1990). In maize root tips, succinate synthesis primarily results from the catabolism of aspartate (Roberts et al. 1992). A brief increase in the amount of succinate (1.5 fold), likely resulting from the deamination of aspartate, also has been observed in hypoxically stressed barley roots (Good and Muench 1993). However, these elevated levels of succinate declined to aerobic levels within 24 h (Good and Muench 1993). Since the breakdown of aspartate has no net energy yield, the value of succinate production under low oxygen conditions in plants has been questioned. However, higher levels of succinate synthesis, relative to lactate fermentation, have been found in leaves of wetland plant species compared with those of dryland crop plants (Menegus et al. 1989). These results suggest that the succinate: lactate ratio within a tissue may be useful as a measure of flooding tolerance, as opposed to the level of succinate itself (Menegus et al. 1989). Succinate production also has been associated with proton consumption and may represent another strategy for avoiding cytosolic acidification (Vanlerberghe et al. 1989; Drew 1996).

Regulation of cytoplasmic pH under anaerobiosis also may involve the production of γ-aminobutyric acid (GABA). The requirement for protons in the conversion of

glutamate to GABA by glutamate decarboxylase (L-glutamic acid + $H^+ \rightarrow$ GABA + CO_2) may help to delay cytoplasmic acidosis (Streeter and Thompson 1972). In an acidic environment, not unlike that acidification associated with an oxygen-free environment, isolated mesophyll cells from asparagus (*Asparagus sprengeri*) produced high amounts of GABA (Crawford et al. 1994). Accumulation of GABA in response to low oxygen stress, reported in both wetland (rice and *Echinochloa*) and non-wetland plant species (maize, rye, barley, wheat, and radish), lend support for the role of GABA synthesis in cytosolic pH regulation (Menegus et al. 1989; Streeter and Thompson 1972). However, studies in maize root tips indicated that GABA production peaked well after the onset of anaerobiosis, suggesting a correlation between high levels of this metabolite and imminent cell death (Roberts et al. 1992). Overall, the functional significance of GABA production in response to flooding conditions has yet to be determined. Recently, Crawford and colleagues (1994) proposed a role for GABA in the intercellular signaling of stress conditions between adjacent cells, as opposed to a direct function for GABA within the cell.

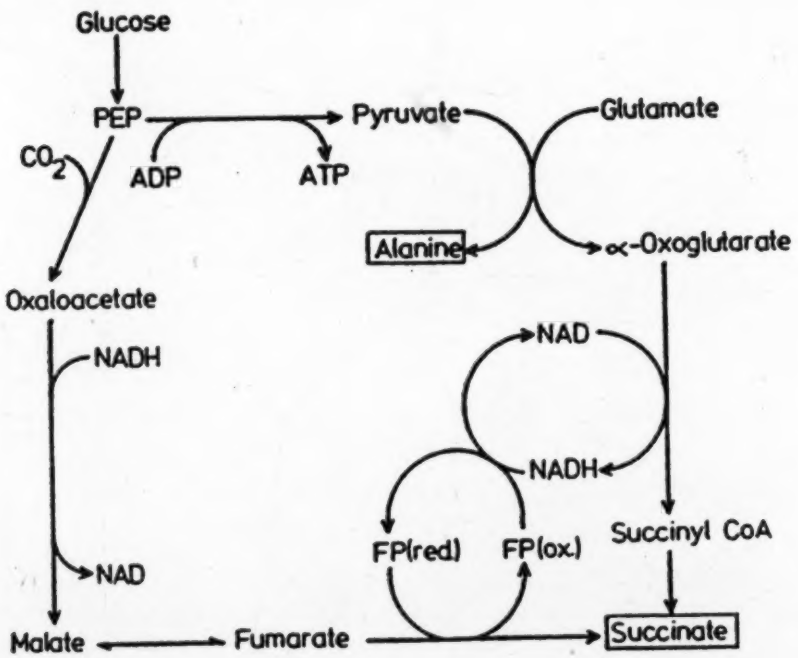

Figure 1.5. A possible pathway for succinate production in plant tissues exposed to anaerobic conditions. PEP, phosphoenolpyruvate; FP, FAD (flavin adenine dinucleotide). From Davies (1980), p. 587.

As with succinate and GABA, elevated levels of alanine also have been detected in various anaerobically stressed plant tissues. Both alanine and succinate synthesis have been associated with the breakdown of aspartate. However, only the amount of alanine declined to low levels after the depletion of aspartate. Succinate levels continued to increase (Streeter and Thompson 1972; Vanlerberghe et al. 1990). The loss of aspartate and concurrent accumulation of alanine occurs in aerial tissues of rice, wheat, and radish (*Raphanus sativus* L.), in roots of barley, and in the N-limited green alga *Selenastrum minutum* (Menegus et al. 1989; Streeter and Thompson 1972; Vanlerberghe et al. 1990; Good and Muench 1993). Under anoxia, alanine production can be prolonged with a pretreatment of ammonium, suggesting that levels of alanine synthesis may be representative of a tissue's nitrogen status (Vanlerberghe et al. 1991; Roberts et al. 1992). The actual role of alanine production in anaerobically stressed tissues, however, remains unclear. Vanlerberghe and associates (1991) hypothesized that the synthesis of alanine may prevent ammonium ions from reaching toxic levels, while sequestering nitrogen within the cell for the production of amino acids when aerobic conditions are restored.

Although elevated levels of succinate, γ-aminobutyric acid, and alanine are detectable at various time points after exposure to reduced oxygen conditions, the functional significance of these metabolites has yet to be elucidated. Further studies focusing on carbon flow within both the shoots and roots of plants grown in the presence and absence of oxygen are necessary to determine absolute rates of metabolite synthesis. A direct comparison between relative levels of all important metabolites under aerobic and anaerobic conditions could lay the foundation for a more detailed explanation of flooding tolerance, integrating the responses of the aerial parts of the plant with those of the root system.

1.5 Gene Expression and the Anaerobic Stress Response

A plant's normal metabolic processes change upon exposure to flooding conditions. The plant reacts by switching from oxidative respiration to various fermentative pathways for continued energy production. This strategic shift requires modifications in the pattern of cellular protein synthesis and gene expression (Figure 1.6).

1.5.1 Effects of flooding on protein synthesis

Sachs and his colleagues (1980) originally determined the impact of flooding conditions on protein synthesis. In the primary roots of maize seedlings, amino acid pulse-labeling experiments revealed a rapid repression of aerobic protein synthesis in a reduced oxygen environment, while synthesis of two novel groups of polypeptides was induced. Within the first hour of anoxia, overall levels of aerobic proteins had decreased, although *in vitro* translation experiments indicated that mRNAs of these proteins remained intact. Synthesis of a group of four transition proteins, each with a molecular weight of approximately 23 kilodaltons, coincided with the translational repression of the aerobic polypeptides. These transition proteins were stable for 48 h following the onset of anoxia; however, a regulatory function for these proteins in the anaerobic stress response has not been identified.

The second phase of protein synthesis begins in maize roots after 90 min of anaerobiosis. From sodium dodecylsulfate 2-dimensional polyacrylamide gel electrophoresis, approximately 20 anaerobic polypeptides (ANPs), including the maize ADH1 and ADH2 proteins, were identified as the major products of protein synthesis. Sachs et al. (1980) reported that synthesis of this novel group of ANPs accounted for more than 70% of the ^{35}S incorporation after a 5 h exposure to anoxia. The levels of these ANPs increased at a constant rate throughout a 3 d period of anaerobiosis. A decline in ANP synthesis was observed later, coinciding with the onset of cell death in the primary root. Similar profiles of ANP synthesis also have been reported in the endosperm, scutellum, anther wall, and coleoptile tissues of maize, when subjected to anoxic treatments (Okimoto et al. 1980).

A scaled-down version of this anaerobic response occurs in the roots of soybean (*Glycine max* L.) (Russell et al. 1990). As seen in maize, the enzyme activity, amount of protein, and mRNA levels of the soybean ADH increase in oxygen-stressed root tissues (Russell et al. 1990). However, only three other ANPs were detected during anoxic treatments. In addition to the smaller number of ANPs, the transcripts of other proteins involved in glycolysis failed to accumulate under flooding conditions (Russell et al. 1990). These findings suggest that the anaerobic response of soybean is less complex than that of maize.

Exposure to anaerobic conditions generally results in the synthesis of a limited number of proteins. This decrease in the overall amount of protein synthesis coincides with previous observations of polyribosome degradation under flooding conditions. Early studies in soybean roots revealed a rapid dissociation of polysomes following the onset of

anoxia (Lin and Key 1967). Upon the return to oxygenated conditions, polysome reassembly occurred, bringing polysome numbers up to 90% of normal levels (Lin and Key 1967). Disruption of polysome structure also has been reported in maize and pea, possibly reflecting preferential translation of a limited group of proteins, as intact aerobic transcripts still reside within oxygen-stressed cells at least 5 h after the onset of anoxia (Ho and Sachs 1989; Sachs et al. 1980; Webster et al. 1991). Changes also occur in polyribosomes, as reflected by altered electrophoretic mobility. This may serve to increase the translational efficiency of ANPs (Bailey-Serres and Freeling 1990). Although polysome dissociation takes place in several different plants, this degradation does not occur under similar conditions in flood tolerant rice and *Echinochloa phyllopogon* (Mocquot et al. 1981; Kennedy et al. 1992). This difference between sensitive and tolerant plants may signify the importance of maintaining a high rate of protein synthesis to survive flooding conditions.

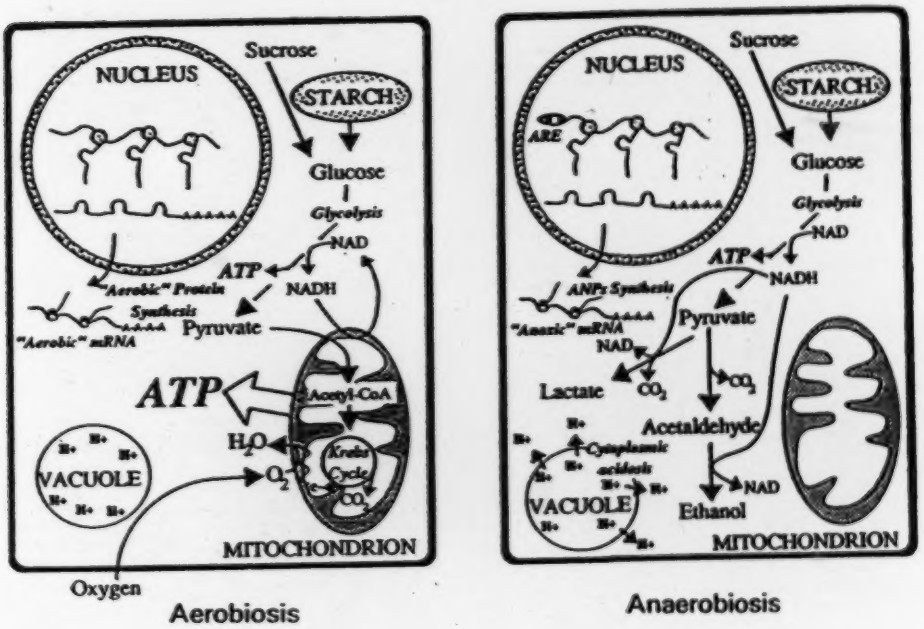

Figure 1.6. A schematic diagram illustrating the biochemical and molecular events occurring at the cellular level under aerobic (left) and anaerobic conditions (right). From Perata and Alpi (1993), p. 12.

1.5.2 Expression of maize ADH proteins under anaerobic stress

Perhaps the best studied example of stress-induced polypeptides in plants is the *Adh1* gene of maize. The importance of *Adh1* gene function in the anaerobic stress response has been demonstrated by the different abilities of wild-type and *Adh1* null mutant seedlings to survive flooding conditions. Several *Adh1*$^-$ mutants have been obtained by an allyl alcohol selection scheme. ADH catalyzes the oxidation of allyl alcohol to acrolein. Acrolein is a toxic substance that poisons *Adh1*$^+$ seedlings, favoring growth of *Adh1* null mutants (Jacobs et al. 1988). Mutants selected in this way succumb to oxygen-stress within a few hours, whereas wild-type seedlings can tolerate a reduced oxygen environment for up to 3 d (Schwartz 1969). The anaerobic inducibility of the maize ADH1 enzyme (EC 1.1.1.1) was initially shown by Hageman and Flesher (1960). Varying amounts of the ADH1 protein have been detected in maize seeds and pollen grains, as well as in root tissues exposed to low oxygen conditions (Freeling and Bennett 1985). The ADH enzyme functions as a dimer, with a molecular weight of 80,000 daltons (Schwartz and Endo 1966; Freeling and Schwartz 1973). This dimerization might occur between two ADH1 molecules, forming a homodimer, or an association with the product of the maize *Adh2* gene may occur, resulting in a heterodimeric enzyme (Freeling 1973; Freeling and Schwartz 1973). The ADH2 protein has been detected in nodes of the tassels and stems of maize plants and is also inducible in root tissues under flooding conditions (Dennis et al. 1985; Sachs et al. 1980).

Although activity levels of both maize ADH enzymes increase in seedlings exposed to low oxygen stress, ADH1 appears to play a more important role in the anaerobic stress response. Mutational analysis indicated that *Adh2* null mutants can tolerate flooding conditions for the same amount of time as their wild-type counterparts (Dennis et al 1985). However, a role for the ADH2 protein cannot be discounted completely, as maize seedlings lacking both the ADH1 and ADH2 proteins succumbed to anoxia sooner than *Adh1*$^-$ single mutants (Dennis et al. 1985). Because of its lower level of activity (1/10 that of ADH1), the contribution of ADH2 to the anaerobic stress response likely goes unnoticed in *Adh1*$^+$ maize seedlings (Freeling and Bennett 1985). Differences in the functional significance of the two enzymes in the flooding response are exemplified further at the mRNA level. Transcript levels of both *Adh1* and *Adh2* increase approximately 50 fold in root tissues within 7 to 10 h after the onset of anoxia (Gerlach et al. 1982; Dennis et al. 1985). The amount of *Adh2* mRNA decreases after 10 h, while *Adh1* transcripts remain at this elevated level for at least 48 h (Gerlach et al. 1982; Dennis et al. 1985). Regardless of the differential expression patterns of these two

genes, the overall increase in the rate of ADH transcription coincides with an accumulation of the ADH protein and induction of enzyme activity under conditions of anaerobic stress.

Several studies of the maize ADH enzyme have explored the effects of manipulating absolute levels of oxygen in the growth medium. Maize root tips generally do not survive more than 24 h of strict anoxia, which results in a temporary acceleration of glycolysis, a transient induction of ADH activity, and a rapid decline in the rate of anaerobic respiration within the root tip cells (Roberts et al. 1984a; Hole et al. 1992; Andrews et al. 1993). However, in nature, soil waterlogging usually brings about a gradual decrease in the availability of oxygen to plant roots. The induction patterns of ADH change if root tips are subjected to a period of hypoxia before the onset of oxygen-free conditions. By sparging the growth solution with 4% oxygen in nitrogen (v/v), root tip cells of maize could tolerate anoxia for more than 96 h (Johnson et al. 1989). The hypoxic pretreatment also resulted in an increase of ADH activity (Johnson et al. 1989). Levels of ADH continued to rise throughout the first 24 h of anoxic conditions, peaking at a level approximately 20 fold higher than that of aerobically grown controls (Johnson et al. 1989). Similar results were obtained when a brief exposure to hypoxia induced ADH activity, which remained high during the subsequent anoxic treatment (Andrews et al. 1993; VanToai et al. 1995). In experiments with maize *Adh1* null mutants, hypoxically pretreated root tips had elevated levels of ADH2 relative to untreated root tips under anoxia (Johnson et al. 1994). Analogous studies in wheat revealed higher levels of ADH induction and increased rates of survival in root tips subjected to hypoxia, prior to an oxygen-free treatment (Waters et al. 1991). Elevated ADH activity levels also have been reported in many other plant species (Table 1.1).

1.5.3 Induction of LDH activity in hypoxic roots

As with ADH, lactate dehydrogenase (LDH; EC 1.1.1.27) activity increases in root tissues upon exposure to a reduced oxygen environment. The LDH enzyme catalyzes the conversion of pyruvate to lactate with the concurrent oxidation of NADH (Figure 1.7). In roots of barley seedlings, a reduction of oxygen concentrations to 3% caused a 20 fold induction of LDH activity after 6 d of hypoxia (Hoffman et al. 1986). Elevated activity levels were detected throughout the entire root system, with the highest levels in root tip cells. Enzyme activity declined once aerobic conditions were restored (Hoffman et al. 1986). Analysis of barley root extracts by native polyacrylamide gel electrophoresis revealed an isozyme profile consisting of five bands, a banding pattern

commonly seen when two *Ldh* genes are present (Hoffman et al. 1986). This pattern is thought to result from the random association of monomeric proteins. The similar isozyme profiles between vertebrates and barley suggest that the barley LDH proteins aggregate to form tetramers (Hanson and Jacobsen 1984; Hoffman et al. 1986). Isolation of two separate barley of LDH cDNAs supported this theory. Sequence comparisons between the individual cDNAs revealed 96% amino acid identity and 92% sequence homology within the coding region (Hondred and Hanson 1990). Using these cDNAs as probes for Northern blots, LDH mRNA levels were shown to increase 8 fold in barley tissues after 2 d of hypoxia (Hondred and Hanson 1990). Hypoxic induction of LDH expression in the roots of barley seedlings occurs at the level of transcription, accompanied by increases in the amount of protein and enzyme activity.

Table 1.1 Levels of ADH Induction under Oxygen Deprivation

Plant Species	Induction	Treatment	Location	References
Petunia hybridia (petunia)	(E) and (R) MND	16 h (H)	roots	Gregerson et al. (1991) Strommer et al. (1993)
Glyceria maxima	2 fold (E)	24 h (A)	roots	Jenkin and ap Rees (1983)
Pisum sativum (pea)	3-4 fold (E)	12 h (A)	roots	Llewellyn et al. (1987)
Oryza sativa (rice)	2-3 fold (E)	48 h (A)	seedlings	Bertani et al. (1980)
Glycine max (soybean)	3 fold (E)	96 h (A)	suspension cultures	Mohanty et al. (1993)

Induction values represent the maximum increases in enzyme activity (E) or mRNA level (R). Treatment refers to the length of exposure to either hypoxic (H) or anoxic (A) conditions, at which time peaks of enzyme activity or transcription were recorded. MND, magnitude not determined. Soybean suspension cultures were derived from hypocotyl tissue.

Similar patterns of LDH induction have been reported in a variety of other plant species. Following a 6 d exposure to hypoxic conditions, LDH activity increased in the root tissues of maize (3 fold induction), wheat (16 fold induction), and rye (80 fold induction) (Hoffman et al 1986). Isozyme profiles from these three species closely resembled those seen in barley (Hoffman et al. 1986). Elevated levels of LDH activity (10 times the aerobic level) also were detected in barley aleurone layers (Hanson and Jacobsen 1984). Both hypoxic and anoxic treatments of rice resulted in a 2 to 3 fold increase in enzyme activity, along with a parallel increase in the amount of enzyme in

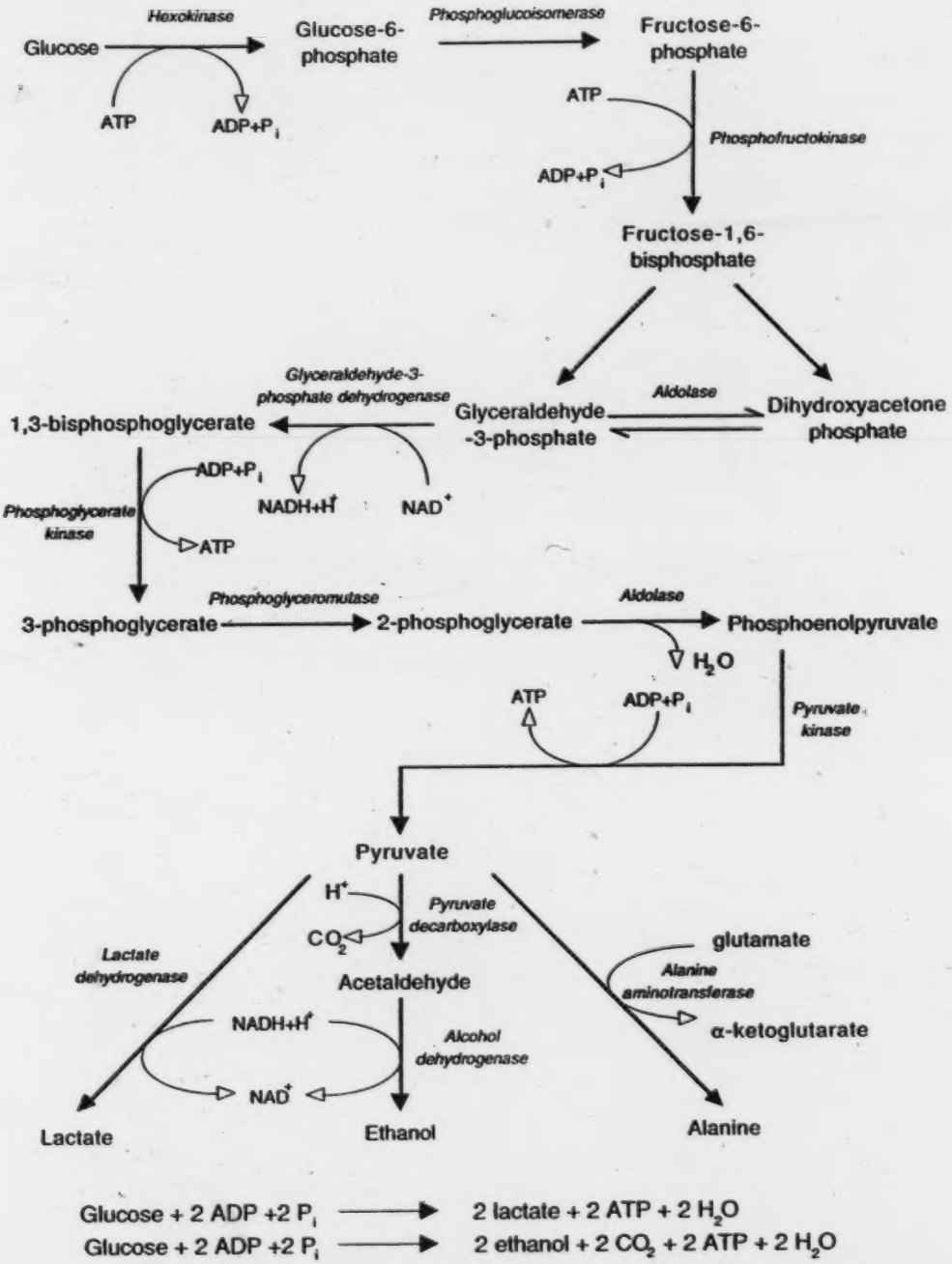

Figure 1.7. The breakdown of glucose by the glycolytic pathway, including 3 anaerobically important fates of pyruvate. Adapted from Mathews and van Holde (1990), p. 447.

the root tissues (Rivoal et al. 1991). Closer examination of the native protein indicated that the rice LDH was as a tetramer, with a molecular weight of 150 kD (Rivoal et al. 1991).

A discrepancy exists between the time course of LDH enzyme induction and lactate production in roots. Accumulation of lactate during the initial stages of anaerobic respiration occurs well before the induction of LDH activity. If the elevated levels of LDH mRNA, protein, and enzyme activity accelerate lactate production, lactate may be metabolized further or secreted out of the cells (Hoffman et al. 1986). The secretion of lactate under hypoxia has been demonstrated in transgenic tomato roots overexpressing the barley LDH cDNA (Rivoal and Hanson 1994). These results, along with evidence of lactate export in maize root tips, suggest that the induction of LDH activity may result in higher rates of lactate production (Xia and Saglio 1992). However, instead of accumulating within the cell and causing acidification of the cytoplasm, the excess lactate may be removed by some kind of hypoxically inducible lactate transporter in the plasma membrane (Rivoal and Hanson 1994). In this type of system, increased lactate production could be used to regenerate NAD^+ for continued glycolysis under low oxygen conditions without harming the plant. Therefore, LDH and lactate production may have roles in both short term and long term flooding tolerance.

1.5.4 Involvement of AlaAT in the anaerobic stress response

Recently, a novel type of anaerobically inducible enzyme involved in amino acid production has been identified. Alanine aminotransferase (AlaAT; EC 2.6.1.2) reversibly catalyzes the reaction between pyruvate and glutamate to yield alanine and α-ketoglutarate (Figure 1.7). Along with its role in amino acid biosynthesis, AlaAT function also has been implicated in carbon shuttling (C_3 units) between mesophyll and bundle sheath cells in C_4 plants (Hatch 1973; Son et al. 1991). The intracellular locations of AlaAT in the photosynthetic leaf tissues of plants include the peroxisomes (90% of activity) and, to a lesser extent, the mitochondria (10% of activity), although other reports also localize AlaAT to the cytosol (Wightman and Forest 1978; Noguchi and Hayashi 1981; Watson et al. 1992). In the non-green tissues of plants, AlaAT activity resides primarily within the mitochondria. Pyridoxal-5-phosphate commonly is associated with aminotransferases. Several plant AlaATs require this cofactor for activity, including the enzymes in broomcorn millet (*Panicum miliaceum*) (Hatch 1973; Son et al. 1991).

Alanine has been found to accumulate in the root tissues of many plant species under reduced oxygen conditions, most notably in pea and barley (Smith and ap Rees 1979; Hoffman et al. 1986). The identification of a hypoxically inducible AlaAT may account for the increased rate of alanine synthesis. Induction of AlaAT activity after a 4 to 5 d exposure to hypoxia has been reported in the roots of maize (2 fold increase), wheat (2 fold), rye (3-4 fold), and barley (4 fold) (Good and Crosby 1989). Closer examination of the AlaAT-2 isozyme in barley roots indicated that the native protein is a homodimer, with a molecular weight of approximately 97 kD (Good and Muench 1992). Elevated levels of AlaAT-2 activity in barley roots were accompanied by corresponding increases in the amount of protein (6 fold induction after 96 h of hypoxia) and mRNA (4 fold increase after 48 h of hypoxia) (Muench and Good 1994). This induction lends support to the role of alanine biosynthesis in a plant's anaerobic response. Unlike ethanol and lactate, alanine accumulates within the cell and may be used as a carbon and nitrogen source for other metabolic processes, such as the synthesis of other amino acids, upon the return of aerobic conditions.

Since their description in maize, many anaerobic polypeptides have been isolated and characterized in a wide variety of plant species (Sachs et al. 1980). The induction ADH, LDH, and AlaAT help to explain the increased production of ethanol, lactate, and alanine by plant root tissues in a reduced oxygen environment. Nearly all of the other ANPs identified in both flood tolerant and sensitive plants have a role in glycolysis or the subsequent catabolism of pyruvate (Tables 1.2, 1.3). These findings further support the theory that the glycolytic pathway and a number of fermentation pathways continue to operate under anaerobic conditions, providing a biochemical basis of plant flooding tolerance.

1.5.5 Transcriptional regulation of stress-inducible proteins

Anaerobic polypeptides within a plant or between different species of plants follow similar patterns of activation under low oxygen conditions. In many cases, elevated mRNA levels precede the observed increases in enzyme activity, suggesting that regulation may occur at the transcriptional level. Preliminary support for this theory comes from alterations in chromatin structure and increased nuclease sensitivity within the maize *Adh1* upstream sequences which coincide with the onset of anaerobiosis (Ferl 1985; Paul et al. 1987). These types of changes in chromatin structure are commonly associated with transcriptionally active genes (Weintraub and Groudine 1976). Closer

Table 1.2 Anaerobically Inducible Proteins from Flood-Tolerant Plants

Enzyme	Plant Species	Induction	Treatment	Location	References
enolase (EC 4.2.1.11)	*Echinochloa phyllopogon* (barnyard grass)	5 fold (R) 5 fold (E)	2 d (A) 5 d (A)	seedlings seedlings	Fox et al. (1995)
enolase	*Oryza sativa* (rice)	8 fold (R) 5 fold (E)	24 h (A) 7 d (A)	seedlings seedlings	Umeda and Uchimiya (1994) Fox et al. (1995)
lactate dehydrogenase (EC 1.1.1.27)	*Oryza sativa*	2 fold (E)	2 d (H)	roots	Mohanty et al. (1993) Ricard et al. (1991)
sucrose synthase (EC 2.4.1.13)	*Oryza sativa*	3 fold (E)	6 d (A)	seedlings	Ricard et al. (1991)

Maximum values of enzyme activity (E) or mRNA accumulation (R) under various exposure times to anoxic (A) or hypoxic (H) conditions are listed in this table.

Table 1.3 Other Inducible Anaerobic Polypeptides in Maize

Enzyme	Induction	Treatment	Location	References
phosphogluco-isomerase (EC 5.3.1.9)	2 fold (E)	72 h (A)	roots	Kelley and Freeling (1984b)
aldolase (EC 4.1.2.13)	6 fold (R)	12 h (A)	HPT root tips	Andrews et al. (1994) Kelley and Freeling (1984a)
	1.48 fold (E)	72 h (A)	roots	Hake et al. (1985)
	20 fold (R)	2-3 h (A)	roots	Dennis et al. (1988)
glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	6 fold (R) 1.5 fold (E)	6 h (A) 24 h (A)	roots roots	Kelley and Freeling (1984b) Russell and Sachs (1989;1992) Bailey-Serres et al. (1988)
phosphoglucomutase	2 fold (E)	48 h (A)	roots	Kelley and Freeling (1984b)
enolase (EC 4.2.1.11)	2-5 fold (R) 4 fold (A)	48 h (A) 48 h (A)	root tips root tips	Lal et al. (1991) Andrews et al. (1994)
pyruvate decarboxylase (EC 4.1.1.1)	15 fold (R)	12 h (H)	root tips	Andrews et al. (1994)
sucrose synthase (EC 2.4.1.13)	20 fold (R)	20 h (A)	primary roots	Springer et al. (1986)

The induction values in this table indicate the peaks in enzyme activity (E) or mRNA level (R) reached during various hypoxic (H) or anoxic (A) treatments. HPT, hypoxically pretreated.

examination of the *Adh1* promoter sequence by deletion analysis delineated a 40 base pair (bp) region necessary for anaerobic induction (Walker et al. 1987a). This anaerobic responsive element (ARE) was further subdivided into regions I (bases -140 to -124, upstream of the transcription start site) and II (bases -113 to -99), deemed essential for anaerobic expression by mutational analysis, whereas mutations within the intervening sequence did not affect *Adh1* inducibility (Walker et al. 1987a). Both the ARE I and II regions contain a core sequence of TGGTT, which also has been found in ANPs of maize and other species (Table 1.4) (Walker et al. 1987a,b).

Table 1.4 Anaerobic responsive element core sequences.

Plant	Gene	Sequence	References
Maize	*Adh1-1S*	$^{-133}$CCTGGTTTCG^{-124} (ARE I)	Ferl and Nick (1987) Walker et al. (1987a)
Maize	*Adh1-1S*	$^{-113}$CGTGGTTTGC^{-104} (ARE II)	Ferl and Nick (1987) Walker et al. (1987a)
Maize	*Adh2-N*	$^{-143}$CGTGGTTTCT^{-134} (ARE I)	Dennis et al. (1985)
Maize	sucrose synthase	$^{+431}$CGTGGTTTGC^{+440} $^{-171}$TCTGGTTTTG^{-162}	Werr et al. (1985)
Maize	aldolase	$^{-68}$GCTGGTTTGT^{-59}	Dennis et al. (1988)
Pea	*Adh*	$^{-101}$TTTGGTTTGT^{-112} (reverse orientation)*	Llewellyn et al. (1987)
Arabidopsis	*Adh*	$^{-153}$TTTGGTTTTG^{-162} (reverse orientation)*	Chang and Meyerowitz (1986)
	Consensus Sequence	TC TC CGTGGTTTGT GT CG	Walker et al. (1987b)

For the sequences listed, the numbers correspond to nucleotide positions upstream of the transcriptional start site. The underlined sequences represent the ARE core sequence. Table modified from Walker et al. (1987b)

*In Arabidopsis and pea, the ARE core was found in the reverse orientation on the complementary DNA strand.

In addition to the identification of regulatory sequence elements, *in vivo* dimethyl sulfate (DMS) footprinting studies uncovered three protein binding sites in the promoter sequence of the maize *Adh1* gene. Two of these binding sites, labeled region A (bases -100 to -108, relative to the transcription start site) and region C (bases -186

to -190), were bound by regulatory factors only during exposure to low oxygen conditions (Ferl and Nick 1987). The third binding site, referred to as region B_1/B_2 (B_1: bases -117 to -120; B_2: bases -138 to -145), lies within the ARE sequence and was occupied in both aerobic and anaerobic environments (Ferl and Nick 1987). The factor(s) binding to the composite B_1/B_2 site undergo a conformational change upon the induction of *Adh1* transcription (Paul and Ferl 1991). Although the association of proteins with regions A and C has been demonstrated under hypoxia, neither sequence is essential for the hypoxic induction of gene expression. However, full transcriptional activation of the *Adh1* promoter was correlated with protein factor binding at all three sites (Paul and Ferl 1991).

Recently, two putative regulatory proteins that bind to portions of the enhancer-like ARE sequence have been identified. Ferl (1990) isolated a major DNA binding protein complex, referred to as ARF-B_2 (ARE binding factor), that interacts with the DNA in the B_2 footprinted region of the maize *Adh1* promoter. ARF-B_2 is thought to consist of multiple components of which only one directly contacts the B_2 sequence (Ferl 1990). Consistent with previous findings regarding the constitutive protein occupation of the B_1/B_2 region of the ARE, ARF-B_2 binding activity was detected under both aerobic and anaerobic conditions (Ferl 1990). The functional significance of ARF-B_2 binding has yet to be determined.

In addition to ARF-B_2, another nuclear protein in maize (GCBP-1) that interacts with two GC- rich sequences in the ARE has been isolated (Olive et al. 1991). Certain mutations within the ARE sequence affect GCBP-1 binding *in vitro*, indicating that the 5' upstream regions of bases -135 to -131 and -120 to -112 represent GCBP-1 binding sites in the *Adh1* promoter (Olive et al. 1991). Along with defining the interaction sites of GCBP-1, mutational analysis also demonstrated the requirement of GCBP-1 binding for significant transcriptional activation of *Adh1* under hypoxic conditions (Olive et al. 1991). The detection of GCBP-1 in both aerobic and induced cells suggests that this protein undergoes some type of post-translational modification before exerting its stimulatory effect on *Adh1* transcription (Olive et al. 1991).

The interaction of regulatory proteins with the ARE for the hypoxic induction of gene expression may be supplemented by the binding of similar types of factors to G-box sequences (5' - CCACGT - 3'). G-box sequence elements have been detected in a variety of environmentally inducible genes (de Vetten and Ferl 1994). Shortened G-boxes (5' - GTGG - 3') are present within some of the dimethyl sulfate footprints of the maize *Adh1* promoter. Protein factors occupying the half G-box elements at positions -99 and -180 of the 5' upstream sequences are only present under hypoxic conditions and may affect

the transcriptional activation of *Adh1* (Ferl and Nick 1987; Paul and Ferl 1991; Ferl and Laughner 1989). Recently, a G-box binding factor (GBF1) has been cloned and characterized in maize (de Vetten and Ferl 1995). Northern blot analysis showed a 6 to 7 fold increase of GBF1 mRNA after a 1 h exposure to hypoxic conditions, but these transcript levels subsequently declined to 4 to 5 times the aerobic levels within 4 to 8 h of hypoxia (de Vetten and Ferl 1995). Comparisons between the timing of mRNA accumulation indicated that GBF1 mRNA levels peaked just prior to the induction of *Adh1* transcription under low oxygen conditions (de Vetten and Ferl 1995). The combination of results from the footprinting and expression studies suggests that G-box binding by GBF1 plays a role in the hypoxic activation of *Adh1* in maize.

In summary, much experimental evidence has been generated in support of the transcriptional regulation of gene expression in a reduced oxygen environment. The exact nature of this regulation has yet to be determined. Walker and colleagues (1987b) speculate that gene activation is achieved through the binding of positive regulatory factors. However, the different kinetics of induction for the various ANPs suggest that the signal transduction pathway governing the hypoxic inducibility of genes may involve a more complex system of regulation.

1.6 The Anaerobic Stress Response in *Arabidopsis thaliana*

Recently, research has focused on the elucidation of the anaerobic stress response in *Arabidopsis thaliana*, which has become a model system for the genetics of higher plants. *Arabidopsis* is a small annual weed and is a member of the Cruciferae (mustard) family. Even though *Arabidopsis* is not an economically important plant, many features make this organism appealing for genetic analysis. Its small size and short life cycle (approximately 6 weeks) permit easy indoor maintenance of large numbers of plants within a small space (Meyerowitz 1989). In *Arabidopsis* seeds, embryos are composed of fewer cells, relative to seeds of larger plants, which means there is a higher probability of successfully mutagenizing the progenitor cells of early flowers by ethyl methanesulfonate (EMS) treatment (Redei 1975; Estelle and Somerville 1986). Self-fertilization occurs in *Arabidopsis*, which would facilitate the isolation of homozygous, recessive, EMS-induced mutants. At the molecular level, *Arabidopsis* has a low DNA content, consisting of approximately 7×10^7 bp per haploid genome (Leutwiler et al. 1984). This small genome size facilitates the mapping and cloning of genes by allowing a good representation of the genome to be obtained with relatively few lambda or cosmid library clones (Meyerowitz 1989; Gepstein and Horwitz 1995). The combination of

these characteristics makes this species very useful in both classical and molecular genetic research.

1.6.1 Induction and regulation of ADH

Alcohol dehydrogenase was among the first of the maize anaerobic proteins to be examined in *Arabidopsis*. Early studies with *Arabidopsis Adh* null mutants demonstrated a role for ADH in the survival of a low oxygen environment. After a 40 h exposure to reduced oxygen conditions, seeds of *Adh* null mutants failed to germinate, whereas wild-type seeds germinated normally (Jacobs et al. 1988). In addition to these mutational experiments, elevated levels of ADH mRNA and enzyme activity have been reported in *Arabidopsis* plantlets following a 15 h anaerobic treatment (Dolferus et al. 1985). The ADH enzyme itself functions as a dimer, with a molecular weight of approximately 87 kD (Dolferus and Jacobs 1984). The protein subunit size, predicted from the *Adh1* cDNA, was 41.2 kD, in agreement with the estimated size of the active enzyme (Chang and Meyerowitz 1986). Sequence comparisons between maize and *Arabidopsis* indicated approximately 73% identity with the coding regions of the maize *Adh1* and *Adh2* genes and approximately 80% identity with the maize ADH1 and ADH2 proteins (Chang and Meyerowitz 1986). Expression of the *Arabidopsis Adh1*, like the *Adh1* gene of maize, occurs primarily in the root tissues of mature plants (Dolferus et al. 1994a). The ADH enzyme is induced in the roots of hypoxically stressed *Arabidopsis* seedlings, although the exact magnitude of this increase has yet to be determined. Low levels of ADH are present in the green, aerial parts of *Arabidopsis* seedlings, and anaerobic induction of the enzyme does not occur in these tissues (Dolferus et al. 1994a,b).

The analogous patterns of *Adh* induction and tissue-specific expression in maize and *Arabidopsis* may denote similarities in gene regulation. Promoter dissections of the maize *Adh1* gene indicated several sequence elements involved in transcriptional regulation, most notably the ARE sequence (positions -140 to -99) (Olive et al. 1991). The GC- and GT-rich domains within the maize ARE also are present in the *Arabidopsis Adh1* promoter region (-160 to -140) but in the opposite orientation (Chang and Meyerowitz 1986). However, experiments with other environmental stresses suggest that this important sequence motif may represent a general stress responsive element (Dolferus et al. 1994a). Along with the identification of an ARE-like element, DMS footprinting studies delineated other regions within this dicot promoter that also may be important in ADH inducibility (Ferl and Laughner 1989; McKendree et al. 1990). Mutations in either of two G boxes in the promoter have no effect on the hypoxic induction of ADH (Figure 1.8); however, induction of the *Arabidopsis Adh1* gene by low

temperature exposure or dehydration is affected to varying degrees (Dolferus et al. 1994b). Deletion of bases -384 to -510 caused a reduction in the anaerobic response of *Adh1*, along with other stress treatments, indicating that this region may contain a binding site for a positive regulatory factor (Dolferus et al. 1994b). A repressor appears to bind upstream of this region, between nucleotides -510 to -964, as the deletion of this sequence results in elevated levels of expression under both aerobic and anaerobic conditions (Dolferus et al. 1994b). Taken together, these footprinting and deletion results indicate that the transcriptional regulation of *Adh1* gene expression in *Arabidopsis* involves several important *cis*-acting sequence elements in various environmental stress responses.

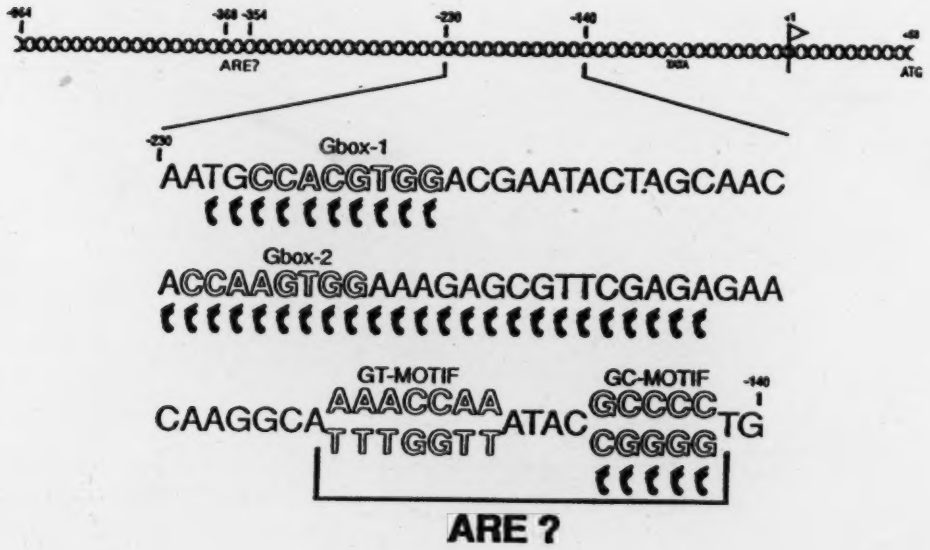

Figure 1.8. Locations of DMS footprinted regions of the *Adh1* promoter in *Arabidopsis*, showing the relative locations of the G box and ARE-like sequence motifs. From Dolferus et al. (1994a), p. 302.

Although genetic experiments indicated the presence of only one *Adh* locus in *Arabidopsis*, a second locus has been identified. The *Adh2* gene shares substantial homology with *Adh1*, at both the DNA (63.1%) and protein (78.6%) levels (Dolferus et

al. 1997a). Unlike *Adh1*, *Adh2* does not exhibit a hypoxic induction pattern (Dolferus et al. 1997a). The *Adh2* gene in *Arabidopsis* is constitutively expressed at low levels in both leaf and root tissues (Dolferus et al. 1997a). The lack of *Adh2* induction under reduced oxygen conditions in *Arabidopsis* roots suggests that *Adh2* may function as a housekeeping gene, producing low amounts of ethanol in the leaves and roots in the presence of oxygen (Dolferus et al. 1997a).

1.6.2 Expression patterns of two pyruvate decarboxylase genes

The hypoxic activation of the *Adh1* gene of *Arabidopsis* prompted a closer examination of the ethanolic fermentation pathway. Questions were raised regarding the activity of the first enzyme in the two step conversion of pyruvate to ethanol, namely pyruvate decarboxylase. As with ADH, *Arabidopsis* has two *Pdc* genes. High expression of *Pdc1* occurs predominantly in the roots of *Arabidopsis* seedlings, whereas *Pdc2* is expressed at low levels in both the root and leaf tissues (Dolferus et al. 1997b). The expression of *Pdc1* is induced under hypoxic conditions, resulting in a 15 to 30 fold increase in *Pdc1* mRNA levels, which peak after 2 to 4 h of hypoxia (Dolferus et al. 1997b). Some form of post-transcriptional regulation may be involved in the activation of *Pdc1* transcription, as the induction at the protein level does not exactly correspond to that at the mRNA level (Dolferus et al. 1997b).

The cloning of the *Pdc1* gene in *Arabidopsis* permitted a detailed analysis of the promoter region to determine if any anaerobic sequence elements were present. The 5' upstream sequences of *Pdc1* contained two GT-motifs at nucleotides -188 to -180 and -174 to -169, relative to the transcriptional start site, and 2 GC-motifs, at positions -165 to -163 and -155 to -151 (Dolferus et al. 1997b). The *Adh1* gene of *Arabidopsis* has only one of each type of sequence motif; however, in both the *Adh1* and *Pdc1* genes, the GT-motifs occur in the same orientation (Dolferus et al. 1994b; 1997b). The two sets of GT- and GC-motifs resemble the number of such elements present in the maize *Adh1* promoter but in the opposite orientation (Walker et al. 1987a; Olive et al. 1991). Comparisons between the two *Pdc* genes of *Arabidopsis* showed that one of the two GT-motifs was missing in the *Pdc2* promoter. The absence of this sequence element in the constitutively expressed *Pdc2* gene raises the question of a possible role for this GT-sequence in the activation of *Pdc1* gene expression. Despite the striking similarities of sequence motifs between these hypoxically inducible genes, the functional significance of these promoter elements remains to be determined.

1.6.3 Duplicate pathways of ethanol production

The parallel increases in transcription of the *Pdc1* and *Adh1* genes of *Arabidopsis* have resulted in speculation regarding the role of two differentially regulated sets of enzymes for a single biochemical pathway. Dolferus and colleagues (1997a,b) hypothesize that the constitutively expressed genes in *Arabidopsis*, *Pdc2* and *Adh2*, are present at low levels in leaves and roots because the need for NAD^+ regeneration for glycolysis by fermentation is overshadowed by the high energy producing tricarboxylic acid (TCA) cycle under aerobic conditions. As levels of oxygen begin to decrease with the onset of anaerobiosis, the *Pdc2* and *Adh2* genes may allow glycolysis to continue during the transitionary phase of decreased TCA cycle function and induction of *Pdc1* and *Adh1* in the root tissues. The prolonged expression of this second set of hypoxically inducible genes may enable the plant to better tolerate a reduced oxygen environment by sustaining glycolytic activity through the oxidation of NADH by ethanolic fermentation (Figure 1.9).

Attempts have been made to determine the molecular mechanism by which this signal transduction pathway is activated. The plant must have some intricate system by which declining levels of oxygen are detected, such that these biochemical changes can be induced. Recently, haemoglobin genes have been isolated in a variety of plant species. In animals, haemoglobin functions as an oxygen carrier in the blood. The identification of these genes in plants raises the possibility of a similar role for haemoglobin in plant tissues (Appleby et al. 1988). Hypoxic conditions result in the induction of one of the haemoglobin genes in *Arabidopsis* (*AtHb1*); mRNA levels increase in both the aerial parts of the plant and the root tissues (Dolferus et al. 1997a). A barley haemoglobin gene also is induced in a low oxygen environment (Taylor et al. 1994).

If some type of oxygen sensor molecule does act in plant tissues, another mechanism must be in place to communicate oxygen levels to all cells within affected tissues. Under anoxic conditions, elevated levels of cytoplasmic calcium have been reported in maize, prior to the induction of gene expression, suggesting that calcium could act as a second messenger in flooding tolerance (Subbaiah et al. 1994a,b; He et al. 1996). Calcium inhibitors were used to determine the effect of calcium levels on the hypoxic induction of *Adh1* gene expression in *Arabidopsis*. Treatments with EGTA (a calcium chelator) and ruthenium red (a calcium channel blocker) reduced transcription levels of *Adh1* under hypoxic conditions (Dolferus et al. 1997a). These results suggest that organellar calcium levels may be part of the triggering device behind anaerobic gene activation.

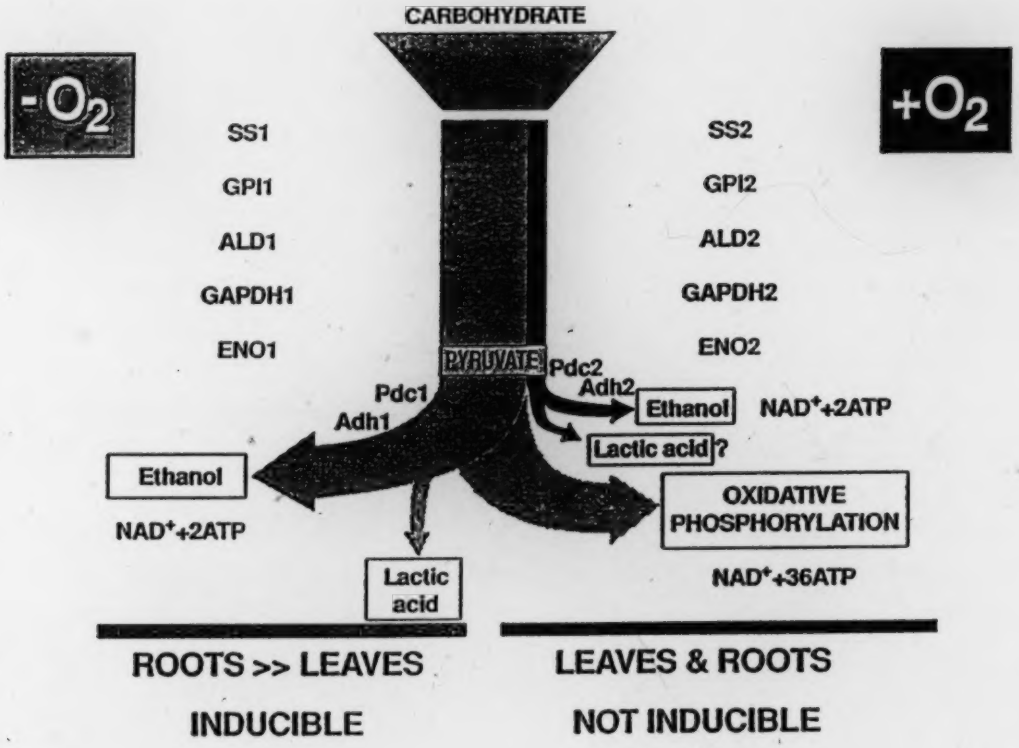

Figure 1.9. A diagramatic representation of the genes involved in carbohydrate metabolism under aerobic (right) and low oxygen (left) conditions in *Arabidopsis*. Enzyme abbreviations: SS, sucrose synthase; GPI, glucose phosphate isomerase; ALD, fructose-1, 6-phosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ENO, enolase. From Dolferus et al. (1997a).

Current research is focusing on the mechanism by which the anaerobic signal transduction pathway is initiated, along with the metabolic changes associated with its activation. The initial steps of the pathway may involve the sensing of molecular oxygen levels by plant haemoglobin molecules, along with changes in intracellular calcium

levels to signal the onset of adverse environmental conditions. The combination of these molecular signals, as well as others that have yet to be identified, would set in motion a series of events ultimately resulting in the activation of gene expression under flooding conditions. The search for other anaerobically important proteins in *Arabidopsis* is also continuing. Although the characterization of *Adh* and *Pdc* expression was well underway in 1993, many other anaerobic proteins, including LDH and AlaAT, had yet to be tested for their inducible expression under hypoxic conditions. Analysis of the patterns of LDH and AlaAT expression, along with the measurement of ADH induction, in the hypoxically stressed roots of *Arabidopsis* were undertaken in this thesis project.

1.7 Goals of this Thesis

The purpose of this project was to characterize some of the biochemical and molecular responses in *Arabidopsis thaliana* under hypoxic conditions. This thesis questioned whether or not three of the anaerobic proteins described in monocot species would be inducible in the roots of mature *Arabidopsis* plants subjected to periods of oxygen deprivation. Specific objectives were to:

1) Assess the effects of low oxygen availability on the enzyme activity, protein, and mRNA levels of alcohol dehydrogenase, lactate dehydrogenase, and alanine aminotransferase in *Arabidopsis*.

2) Clone and characterize the *AlaAT* gene from *Arabidopsis* to determine if the *AlaAT* promoter contains any anaerobic sequence elements.

1.8 References

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2 Induction of alcohol dehydrogenase and lactate dehydrogenase gene expression in *Arabidopsis* roots under hypoxic conditions

2.1 Introduction

The pathways of lactate and ethanol production by LDH and ADH respectively have been well documented as part of the anaerobic stress response in several plant species. The reactions catalyzed by these enzymes share one thing in common. The breakdown of pyruvate is accompanied by a concomitant regeneration of NAD^+, which permits the continuation of anaerobic glycolysis. Under conditions of limited oxygen availability, the short term increase in lactate production likely occurs as a result of LDH induction. The initial burst of lactate synthesis is followed by elevated levels of ethanol fermentation through an increase ADH activity, as proposed by the Davies-Roberts hypothesis (Davies et al. 1974).

Recent evidence suggests that the activation of both ADH and LDH is important in both short and long term responses to flooding. Increases in ADH activity have been reported in many plants including maize (20 fold induction) and rice (2 to 3 fold) during periods of oxygen deprivation (Johnson et al. 1989; Mohanty et al. 1993). Prolonged exposure to low oxygen conditions also results in elevated levels of LDH activity in many monocots (Christopher and Good 1996; Hoffman et al. 1986; Good and Crosby 1989). For the most part, these patterns of hypoxic induction also occur at the protein and mRNA levels for ADH and LDH (Hondred and Hanson 1990; Christopher and Good 1996).

To assess the inducibility of ADH and LDH in *Arabidopsis*, the activity levels of each enzyme were measured under both aerobic and hypoxic conditions. Preliminary results indicated that exposure to a reduced oxygen environment increased ADH activity in *Arabidopsis* plantlets (Dolferus et al. 1985). Since the inducibility of ADH in *Arabidopsis* has been established, this chapter describes the magnitude of the increases in enzyme activity, the amount of protein, and level of mRNA in roots of *Arabidopsis* plants exposed to hypoxic conditions. For LDH, enzyme activity, protein, and transcript levels were compared in uninduced and induced root samples to determine if LDH expression was also inducible under hypoxic conditions. Determination of the induction kinetics of these enzymes facilitated comparisons with other plant species and provide a deeper understanding of the compounds of the anaerobic stress response in *Arabidopsis thaliana*.

2.2 Materials and Methods

2.2.1 Plant Growth Conditions

Seeds of the Columbia (wild type) strain and ADH null mutants (R001) of the *Bensheim* race of *Arabidopsis thaliana* (L.) Heynh (Lehle seeds, Round Rock, Texas) were planted in moist vermiculite, germinated, and grown in a growth chamber for 21-28 d at 22°C with a 16 h photoperiod and a light intensity of 200 $\mu mol/m^2/s$ (Jacobs et al. 1988). Roots of individual plants were then washed free of vermiculite and inserted into plastic rings, such that the bottom leaves of each plant rested on the surface of each ring. These rings were inserted into small holes (7 mm in diameter) cut into a black plexiglass board. This board acted as the lid of a 20 L aquarium, which was painted black to limit algal growth in the nutrient media. The plants were positioned such that the stem and leaves rested on top of the board while the roots hung below in the water. *Arabidopsis* seedlings were grown hydroponically in a half-strength Hoagland's solution (Appendix 5.1), with air continuously being bubbled into the nutrient solution, for 14-21 d. The plants were induced by sparging the growth solution with 100% nitrogen instead of air. Under aerobic conditions, oxygen levels were approximately 9.1 mg/L, whereas under hypoxic conditions, the concentration of oxygen dropped to 0.8 to 1.0 mg/L.

2.2.2 Extraction procedure

Root tissue (0.5-0.7 g FW) was ground (with sand) using a mortar and pestle in an extraction buffer comprised of 50 mM Tris-HCl (pH 8.5), 10% (v/v) glycerol, 10 mM sodium borate, and 10 mM dithiothreitol (DTT) (Hoffman, Bent, and Hanson 1986). Approximately 1.4 ml of buffer was added per gram of root tissue. The mixture was centrifuged for 10 min in a microfuge and placed on ice. The supernatant subsequently was assayed for ADH and LDH activity.

2.2.3 Assay of ADH Activity

Levels of ADH activity were determined by the rate of conversion of ethanol to acetaldehyde (Appendix 5.2.1). Activity of ADH was measured by monitoring the rate of NADH production in the reaction mixture at 340 nm. In a total volume of 1 ml, 50 µl of root extract was added to a reaction buffer containing 0.1M Tris-HCl (pH 9.0), 0.1 M ethanol, and 1 mM NAD (Hanson and Jacobsen 1984). Reactions were carried out for 2 min at 23°C, over which time the optical density of the solution increased. Previous experiments indicated that in the absence of ethanol, the reduction of NAD^+ did not occur

(Good and Sprysak, unpublished results). One international unit (IU) of ADH activity was defined as the amount of enzyme catalyzing the formation of 1.0 µmol of product per minute at 23°C.

2.2.4 Assay of LDH activity

The LDH reaction was assayed in the pyruvate to lactate direction with activities determined by measuring NADH oxidation spectrophotometrically at 340 nm (Appendix 5.2.2). The reaction buffer contained 0.15 M Tris-HCl (pH 8.0), 0.1 mM NADH, and 2.5 mM 4-methylpyrazole hydrochloride (to inhibit any ADH activity in the root extract) in a final volume of 1 ml (Davies et al. 1974; Hoffman et al 1986). Initial absorbance readings were taken of the reaction buffer mixed with 200 µl of root extract to determine background levels of NADH oxidation. The LDH reaction was initiated by adding sodium pyruvate (5 mM final concentration in 1 ml total volume) to the mixture. The absorbance was monitored over a 2 min time period. One international unit (IU) of LDH activity was defined as the amount catalyzing the formation of 1.0 µmol of product per minute at 23°C. Stability tests also were carried out for LDH by assaying the root extracts at various times after extraction. The *in vitro* stability of the *Arabidopsis* LDH enzyme was tested by incubating extracts from induced root samples (48 h of hypoxia) on ice for 0, 1, and 6 h periods and before assaying for LDH activity. Various combinations of DTT (10 mM), BSA (5 mg/ml), and sodium borate (10 mM) were added to the extraction buffer prior to the grinding of the root samples.

2.2.5 Protein measurements

The Bradford assay (Bradford 1976) was used to determine the concentration of total protein in each of the root extracts. Various known concentrations of BSA (bovine serum albumin) were measured at 595 nm to create a standard curve, from which protein concentrations of the extracts were calculated.

2.2.6 Non-denaturing gel electrophoresis

Native gel electrophoresis was performed in mini slab gels (1.5 mm thickness), as described by Hanson and Jacobsen (1984) (Protean Electrophoresis system from Bio-Rad). The separation gel consisted of 7% acrylamide-0.19% bisacrylamide, 0.38 M Tris-HCl (pH 8.9), and 10% (w/v) sucrose and was polymerized with 0.4 µl/ml TEMED and 0.33 mg/ml ammonium persulfate. The stacking gel contained 5% acrylamide - 0.13% bisacrylamide, 60 mM Tris-HCl (pH 6.8), and 10% (w/v)

sucrose. The stacking gel was polymerized in the light with 0.6 µl/ml TEMED and 16 µg/ml riboflavin. Prior to loading, protein samples of 15 and 20 µg (for ADH and LDH, respectively) were mixed in one-fifth volume of gel loading dye (65 mM Tris-HCl [pH 8.0], 35% [v/v] glycerol, and 0.1% [w/v] bromophenol blue). Native gels were run in a buffer composed of 5 mM Tris-HCl (pH 8.3) and 38 mM glycine at constant voltage (30 V) for 12-14h at 4°C.

2.2.7 Stain for ADH activity

Native gels were stained for ADH activity, according to the procedure outlined by Schwartz (1966). Gels were incubated at room temperature for 20 min in a solution consisting of 0.1 M Tris-HCl (pH 8.0), 7% (v/v) ethanol, 0.45 mM NAD, 0.25 mM nitroblue tetrazolium chloride, and 0.16 mM phenazine methosulfate. The native gels then were incubated at 37°C for 1 h in this stain solution or until dark blue bands representing ADH activity appeared on the gels.

2.2.8 Stain for LDH activity

LDH activity was visualized on non-denaturing gels using a stain described by Hanson and Jacobsen (1984). The LDH stain consisted of 0.15 M Tris-HCl (pH 8.0), 0.1 M lithium L(+) lactate, 2 mM magnesium chloride, 0.12 mM nitroblue tetrazolium chloride, 0.1 mM phenazine methosulfate, and 2.5 mM 4-methylpyrazole hydrochloride. Native gels were incubated in this solution for 20 min at room temperature and then were transferred to a 37°C incubator for 1 h or until dark blue bands representing LDH activity appeared on the gels.

2.2.9 Denaturing gel electrophoresis of protein samples

Electrophoresis of denaturing gels was performed using 8% (w/v) SDS-PAGE mini slab gels as described by Laemmli (1970). The stacking gel consisted of 5% acrylamide - 0.13% bisacrylamide, 110 mM Tris-HCl (pH 6.8), and 0.1% SDS and was polymerized by adding 32 mg/ml ammonium persulfate and 0.5 µl/ml TEMED. The separation gel contained 8% acrylamide - 0.2% bisacrylamide, 350 mM Tris-HCl (pH 8.7), and 0.1% SDS. The gel was polymerized with 32 mg/ml ammonium persulfate and 0.5 µl/ml TEMED. Prior to loading, the protein samples (20 µg) were mixed with a denaturation buffer composed of 0.25 M Tris-HCl (pH 6.8), 5% (v/v) glycerol, 5% SDS, 2.5% (v/v) 2 - mercaptoethanol, and 0.1% (v/v) bromophenol blue, and the samples were boiled for 5 min. A mixture of proteins of known molecular weight also

were run on these SDS gels for estimating subunit sizes of the *Arabidopsis* proteins. The prestained broad range SDS-PAGE standards from Bio-Rad included myosin (200 kD), β-galactosidase (116.25 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD), and aprotinin (6.5 kD). Denaturing gels were run at room temperature for 1-2 h at 100 V in a 5 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS buffer.

2.2.10 Western blotting and antibody detection

Proteins from both denaturing and non-denaturing gels were transferred to nitrocellulose membranes by electroblotting, according to the manufacturer's specifications (Trans-blot electrophoretic transfer cell from Bio-Rad). Both types of gels were equilibrated for 30 min in electroblot buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, and 20% [v/v] methanol). Electroblotting of proteins to nitrocellulose was performed at 4°C for 5 h at 60 V. Protein detection by antibody staining was adapted from Good and Crosby (1989). The nitrocellulose membranes were incubated at 4°C for a minimum of 2 h in TBST (10 mM Tris-HCl [pH 8.0], 150 mM sodium chloride, and 0.5% [v/v] Tween 20) containing 5% skim milk powder. These membranes were incubated at 37°C for 1 h in the TBST/milk solution with the primary ADH (1:4000 dilution) or LDH (1:750 dilution) antibody (Good and Crosby 1989; Hoffman and Hanson 1986). The blots were washed three times (5 min each) at room temperature in the TBST/milk solution. The membranes then were incubated at 37°C for 1 h in the TBST/milk solution containing the secondary goat anti-rabbit IgGs (1:7500 dilution) linked to alkaline phosphatase (Promega) and were washed as above. An alkaline phosphatase buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M sodium chloride, and 5 mM magnesium chloride) containing 0.3 mg/ml nitroblue tetrazolium chloride and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was used to develop the blots.

In addition to the alkaline phosphatase method of protein detection, the streptavidin-horseradish peroxidase procedure also was performed, according to the manufacturer's specifications (ECL protein biotinylation system from Amersham). The blots were blocked for a minimum of 2 h in the TBST/milk solution, incubated with the primary antibody, and washed as previously described for the alkaline phosphatase method. The membranes then were incubated for 1 h at room temperature in a PBST solution of pH 7.5 (80 mM di-sodium hydrogen orthophosphate dodecahydrate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, and 0.1% [v/v] Tween 20) with the secondary horseradish peroxide conjugate (1:10,000 dilution) and were

washed three times (5 min each) in PBST. The chemiluminescent substrates were incubated with the membranes for 30 s to 1 min, and then the blots were exposed to film.

2.2.11 Preparation of total RNA and mRNA

Root tissue (2-2.5 g) was harvested from *Arabidopsis* seedlings (5-6 weeks old) growing under either aerobic or hypoxic conditions. The roots were washed, blotted dry, and quickly frozen in liquid nitrogen. Then total RNA was extracted, using a procedure adapted from Yeh et al. (1990). Frozen root tissue was ground to a fine powder in liquid nitrogen, using a mortar and pestle. This powder was mixed with 2 volumes (v/v) of guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% [w/v] lauryl sarcinosate, and 0.1 M 2-mercaptoethanol) and homogenized with a polytron (IKA-VIBRAX VXR from Interscience). The mixture was centrifuged at 9,500 rpm for 20 min at 4°C, and the supernatant was filtered through sterile miracloth. The supernatant was extracted with 1 volume of phenol/chloroform/isoamyl alcohol (25/24/1) and then again with 1 volume of just chloroform/isoamyl alcohol (24/1) to remove cellular proteins. RNA was precipitated by adding a one-tenth volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol. The resulting mixture was incubated at -20°C for a minimum of 2 h. RNA was pelleted in a microfuge at 13,200 rpm for 30 min at 4°C. The RNA pellets were resuspended in sterile diethyl pyrocarbonate (DEPC) - treated water. After dissolving the pellets in DEPC-water, an equal volume of 6 M lithium chloride was added and incubated overnight at 4°C to precipitate the RNA a second time, for the removal of contaminating DNA and polysaccharides. The RNA was pelleted as described above. The pellets were resuspended in sterile DEPC-treated water.

For the isolation of messenger RNA (mRNA), the above procedure for the extraction of total RNA from root tissue was followed to the end of first RNA precipitation step. The lithium chloride precipitation was omitted. Instead, the RNA samples were mixed with a biotinylated-oligo (dT) probe and then were added to streptavidin-paramagnetic particles from a small scale mRNA isolation kit (PolyAT tract mRNA Isolation System IV from Promega). The polyA$^+$ RNA preparations were carried out according to the manufacturer's specifications. After a series of washes, the mRNA was eluted from the streptavidin-paramagnetic particles with RNase-free water.

2.2.12 RNA gels and Northern blot analysis

RNA samples from both aerobic and hypoxically induced roots were subjected to agarose gel electrophoresis. Prior to loading, 5.5 µl of RNA (10 µg of total RNA or 1.0 µg of mRNA, based on OD_{260} readings) was mixed with a denaturation buffer composed of 2.5 µl of 10X MOPS buffer (0.4 M morpholinopropanesulfonic acid, 100 mM sodium acetate, and 10 mM EDTA, with the pH brought up to 7.0 with sodium hydroxide), 4.5 µl of 37% formaldehyde, and 10 µl of deionized formamide to a final volume of 25 µl. The denatured RNA samples then were electrophoresed through a 1.2% agarose gel containing 6% formaldehyde and 1X MOPS buffer overnight at 30 V. A capillary blotting procedure was used to transfer the RNA from the gel to a nylon membrane (GeneScreen Plus from Dupont) according to the method described by Selden (1989). Membranes were baked at 80°C for 2 h.

For Northern blots made with total RNA samples, methylene blue staining was performed to visualize the RNA, as outlined by Sambrook et al. (1989). The membranes were briefly wet in 1X SSPE (0.15 M sodium chloride, 10 mM sodium dihydrogen orthophosphate, and 1 mM EDTA) and then were transferred to a methylene blue solution (0.02% [w/v] methylene blue and 0.5 M sodium acetate [pH]) for 10 min. After blue bands appeared, the membranes were destained in 20% (v/v) ethanol for 5-10 min. The stain was removed by washing the membranes in a 0.2X SSPE, 1% SDS solution for 15 min.

The resulting Northern blots were hybridized at 42°C with 10 to 20 ng of the appropriate radioactively labeled probe (1 to 5 X 10^8 cpm/µg) for 12-16 h in 5X SSPE, 50% (v/v) deionized formamide, 5X Denhardt's solution (2 mg/ml Ficoll 400, 2 mg/ml polyvinylpyrrolidone, 2 mg/ml bovine serum albumin), 125 µg/ml sonicated salmon sperm DNA, and 10% (w/v) dextran sulfate. Subsequently, the blots were washed twice at room temperature for 15 min each in 2X SSPE, once in 2X SSPE, 2% SDS at 65°C for 45 min, and once in 1X SSPE at 65°C for 1 h. Blots were exposed to X-ray film for 1-16 h at -80°C.

2.2.13 Densitometric analysis

Quantification of band intensities on both Northern and Western blots was performed using the GS-670 scanning densitometer (Bio-Rad) and the Molecular Analyst software (Version 1.4 from Bio-Rad).

2.3 Results

2.3.1 Induction of ADH and LDH activity by hypoxia

The activities of both ADH and LDH increased in the tissues of *Arabidopsis* plants upon exposure to low oxygen conditions. Levels of ADH activity continued to rise for 96 h after the onset of hypoxia to 5 to 6 times the aerobic level (Figure 2.1). Although levels of LDH activity were lower than those of ADH, a 2 fold induction of LDH activity also was observed (Figure 2.2). Christopher and Good (1996) reported a reduction in the *in vitro* stability of the hypoxically induced form of the LDH enzyme in maize. In light of these results, the stability of the *Arabidopsis* LDH was tested over time. Hoffman and colleagues (1986) found that the composition of the extraction buffer affected the levels of LDH activity in barley roots under hypoxia. The addition of BSA (5 mg/ml) and sodium borate (10 mM) to an extraction buffer containing Tris-HCl (50 mM, pH 8.5) and DTT (10 mM), enhanced the *in vitro* stability of the barley LDH (Hoffman et al. 1986). Various combinations of these components were included in extraction buffers used to prepare *Arabidopsis* root extracts. The level of LDH activity remained fairly constant over time, regardless of the composition of the extraction buffer (Table 2.1). The root samples of *Adh* null mutants (R001) exhibited a similar increase in LDH activity (Figure 2.2), while ADH levels were negligible (Figure 2.1). These patterns of enzyme activation also were observed on native polyacrylamide gels stained for either ADH or LDH activity (Figure 2.3). A single activity band appeared for each of the two enzymes, suggesting that in each case, only one isozyme is induced by hypoxia in *Arabidopsis* roots. Since the LDH induction profiles of the R001 mutants closely resembled those of wild-type plants, only wild-type plants were used in subsequent experiments.

2.3.2 Immunological detection of the ADH and LDH proteins

Levels of ADH and LDH proteins were monitored under hypoxic conditions to determine if the elevated enzyme activities were associated with increased amounts of the proteins. From Western blots of SDS gels, a single ADH protein band was detected in root extracts using a barley polyclonal antibody (Good and Crosby 1989). Comparisons with blots stained with pre-immune serum revealed a faint band of a similar size, probably resulting from low levels of the rabbit ADH protein in the serum. The intensity of this band on antibody stained blots was significantly greater than that seen on pre-immune blots; no other overlapping bands were detected between the two sets of blots.

These combined results suggested that the barley antibody was detecting the *Arabidopsis* ADH protein.

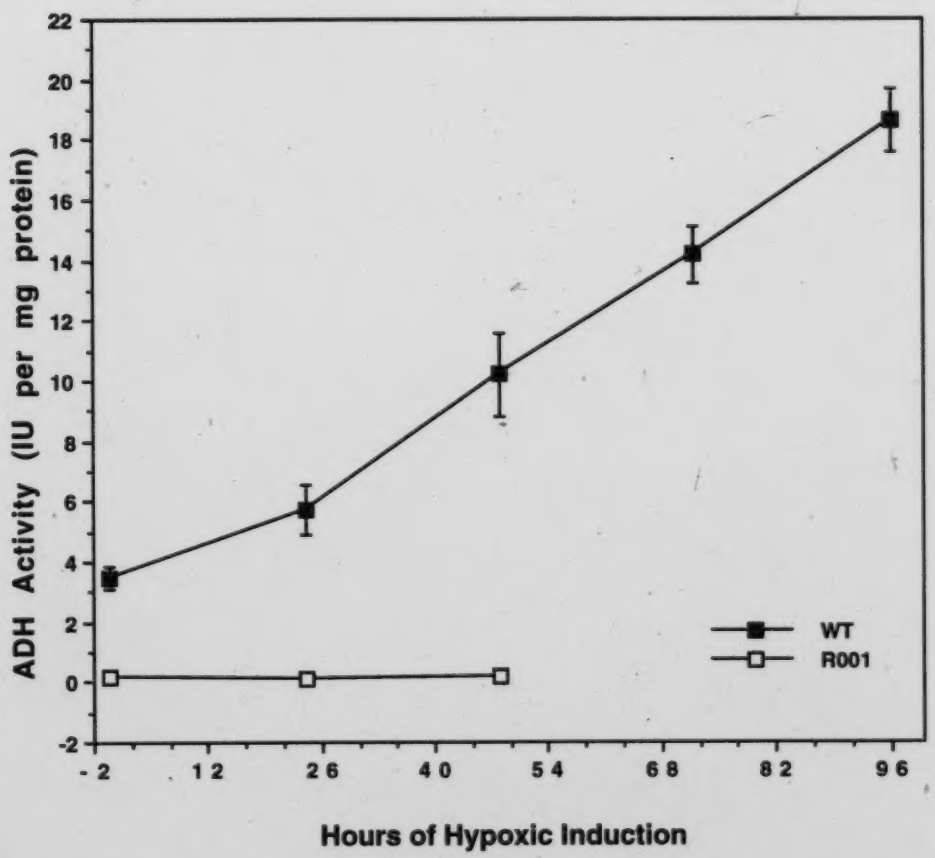


Figure 2.1. Levels of ADH enzyme activity in roots of wild-type (WT) and R001 (ADH^-) *Arabidopsis* seedlings exposed to hypoxic conditions. Activity levels were determined by spectrophotometric enzyme assays. The value at each time point represents the mean of the data obtained from a single experiment (n=3; ± standard error). Experiments were replicated three times.

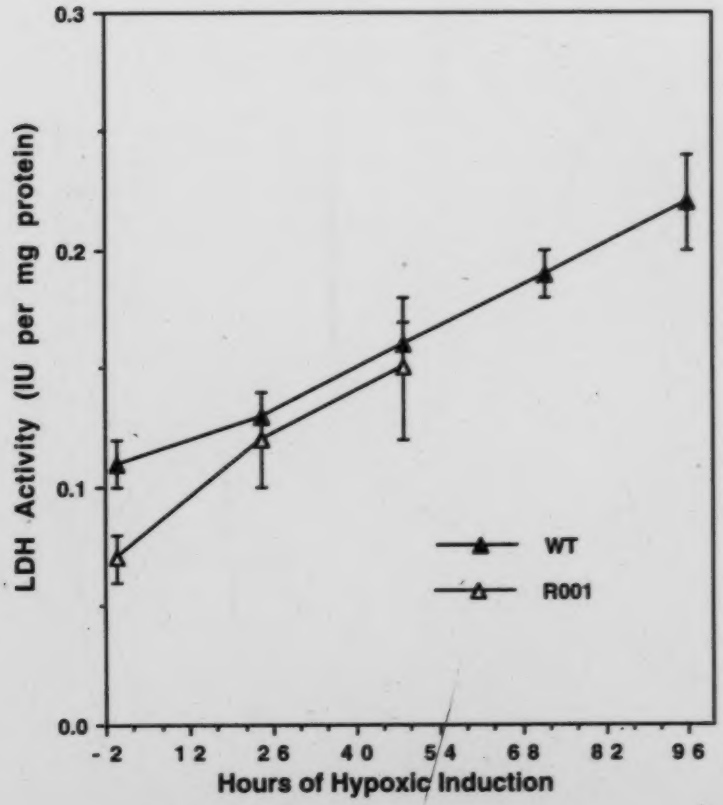


Figure 2.2. Activity levels of LDH in the roots of wild-type (WT) and R001 (ADH^-) *Arabidopsis* plants subjected to conditions of hypoxia. Activity of LDH was monitored using spectrophotometric assays. The value at each time point is the mean (± standard error) of the data obtained from roots of three separate plants. Experiments were replicated three times.

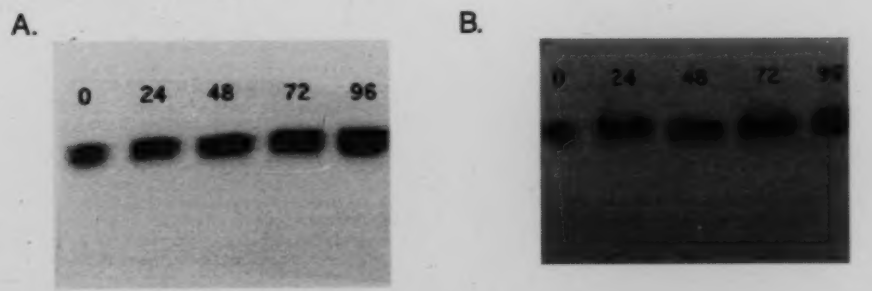


Figure 2.3. Native polyacrylamide gels stained for A. ADH activity and B. LDH activity. Numbers above the bands indicate the hours of hypoxic induction.

Table 2.1. Stability Tests of LDH

Extraction Buffer Supplements	Initial Activity[I]	1 h[S]	6 h[S]
DTT (10 mM)	0.31 (0.02)	0.33 (0.01)	0.30 (0.02)
DTT (10 mM) and sodium borate (10 mM)	0.30 (0.01)	0.30 (0.02)	0.29 (0.02)
DTT (10 mM), BSA (5 mg/ml), and sodium borate (10 mM)	0.29 (0.01)	0.31 (0.02)	0.28 (0.03)

Values in the table indicate enzyme activity levels, measured in IU (international units) per mg of protein in the root extract. The numbers in brackets represent the standard error (n = 2; the third sample did not have detectable levels of activity). In each case, the starting extraction buffer consisted of 50 mM Tris-HCl (pH 8.5) and 10% (v/v) glycerol.

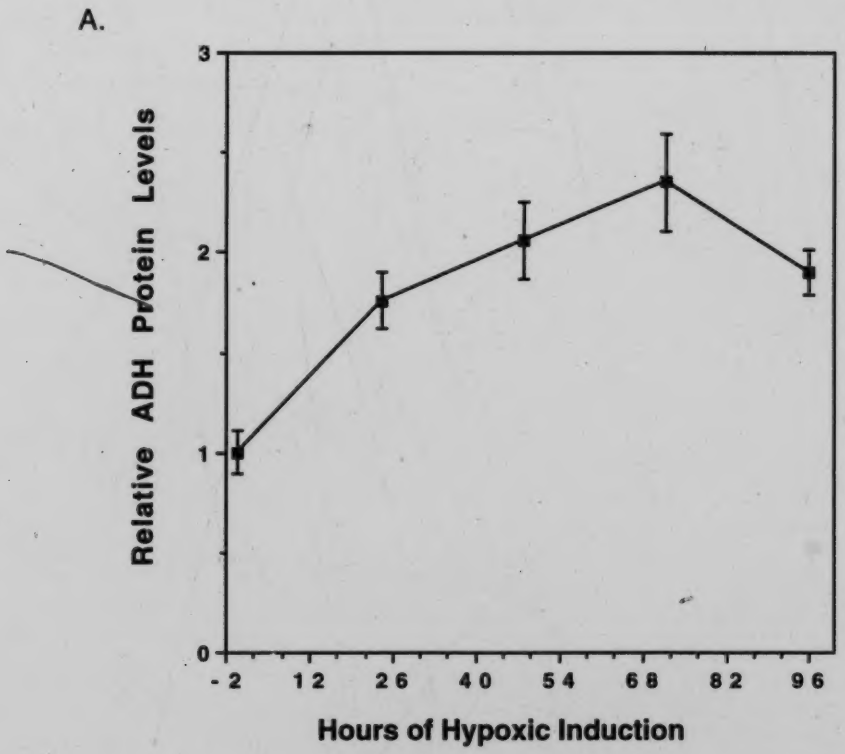
[I] Initial activity refers to the level of enzyme activity measured immediately after extract preparation.

[S] These values represent the enzyme activities detected 1 h and 6 h after the root samples had been homogenized. Samples were incubated on ice during this time period.

Elevated amounts of the ADH protein were observed in root extracts from *Arabidopsis* plants grown under hypoxic conditions. Levels of the ADH protein steadily increased to 2 to 4 times the aerobic levels in the first 48 h of hypoxia. After reaching this plateau, the amount of protein remained reasonably constant up to 96 h, which marked the duration of the experiment (Figure 2.4). The estimated size of this protein band, based on comparisons with the prestained SDS molecular weight standards on denaturing gels, was approximately 45 kD. The estimation is consistent with the 41.2 kD subunit size predicted by Chang and Meyerowitz (1986), along with the estimated size of the functional ADH dimer (87 kD), as determined by Dolferus and Jacobs (1984). These results indicate that the increase in ADH activity is matched by an accumulation of the ADH protein in *Arabidopsis* roots under hypoxia.

Similar experiments were performed to measure any changes in the amount of LDH protein produced under hypoxic conditions. The results of LDH antibody staining with the barley polyclonal antibody (Hoffman and Hanson 1986) were not as clear-cut as with ADH. Comparisons between pre-immune and antibody stained Western blots

revealed a large number of background bands detected by the barley antibody, making identification of the LDH protein band difficult. These background problems were



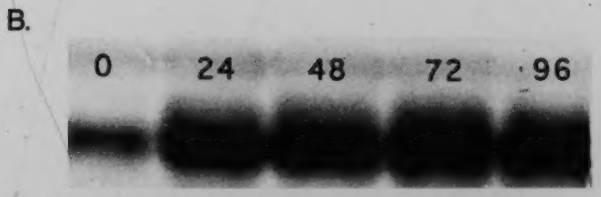


Figure 2.4. Accumulation of the ADH protein in *Arabidopsis* roots under hypoxic conditions.

A. Protein concentrations were measured by densitometry of SDS immunoblots. Aerobic levels were arbitrarily set 1. The value at each time point represents the mean of the data obtained from three separate plants (± standard error). Experiments were replicated three times.

B. SDS immunoblot probed with the barley ADH polyclonal antibody. The numbers across the top of the blot represent the hours of hypoxic exposure.

augmented by the relatively low amounts of LDH synthesized in the root tissue, further preventing accurate quantification of the amounts of protein by densitometry. Despite the poor quality of the immunological detection of LDH, an increase in protein levels was detectable protein levels under low oxygen conditions (Figure 2.5). The LDH protein band consistently migrated faster than the ovalbumin size standard and somewhat slower than carbonic anhydrase, indicating that the LDH protein subunit was approximately 40 kD. This size estimate is similar to that of the barley LDH, which has also a subunit molecular weight of approximately 40 kD (Hondred and Hanson 1990).

Figure 2.5. SDS immunoblot of LDH protein levels in roots of *Arabidopsis* under hypoxic conditions. This SDS-PAGE immunoblot was probed with barley anti-LDH antiserum. The numbers across the top of the blot indicate the hours of hypoxic induction.

2.3.3 Transcriptional induction of ADH and LDH

Upon learning of the hypoxic induction of enzyme activity and protein synthesis for ADH and LDH in *Arabidopsis* roots, the next step was to examine the transcript levels of these two proteins under both aerobic and reduced oxygen conditions. Northern blots made using equal amounts of total RNA from uninduced and induced root samples were probed with a 2.5 kb SstI/HindIII fragment from plasmid jAt3011, which contains a portion of the *Adh1* coding region from *Arabidopsis* (Chang and Meyerowitz 1986). The resulting 1.45 kb *Adh1* transcript accumulated to levels 30 to 35 fold higher than aerobic levels in wild-type *Arabidopsis* roots after a 48 h hypoxic treatment (Figure 2.6). Both methylene blue staining (Section 2.2.12) of Northerns prior to probing and subsequent hybridization with an actin probe from *Arabidopsis*, after probing with *Adh1* served as controls for equal loading (Figure 2.7). Similar increases in ADH mRNA levels have been detected in callus cultures of *Arabidopsis*, made from germinating seeds, under low oxygen conditions (Dolferus et al. 1985).

Elevated levels of the LDH transcript were also detected in induced root samples. A 1.1 kb PstI/SstI fragment derived from an expressed sequence tag cDNA of the *Arabidopsis* LDH (plasmid vector pZL1) was used as a probe for Northern blot analysis

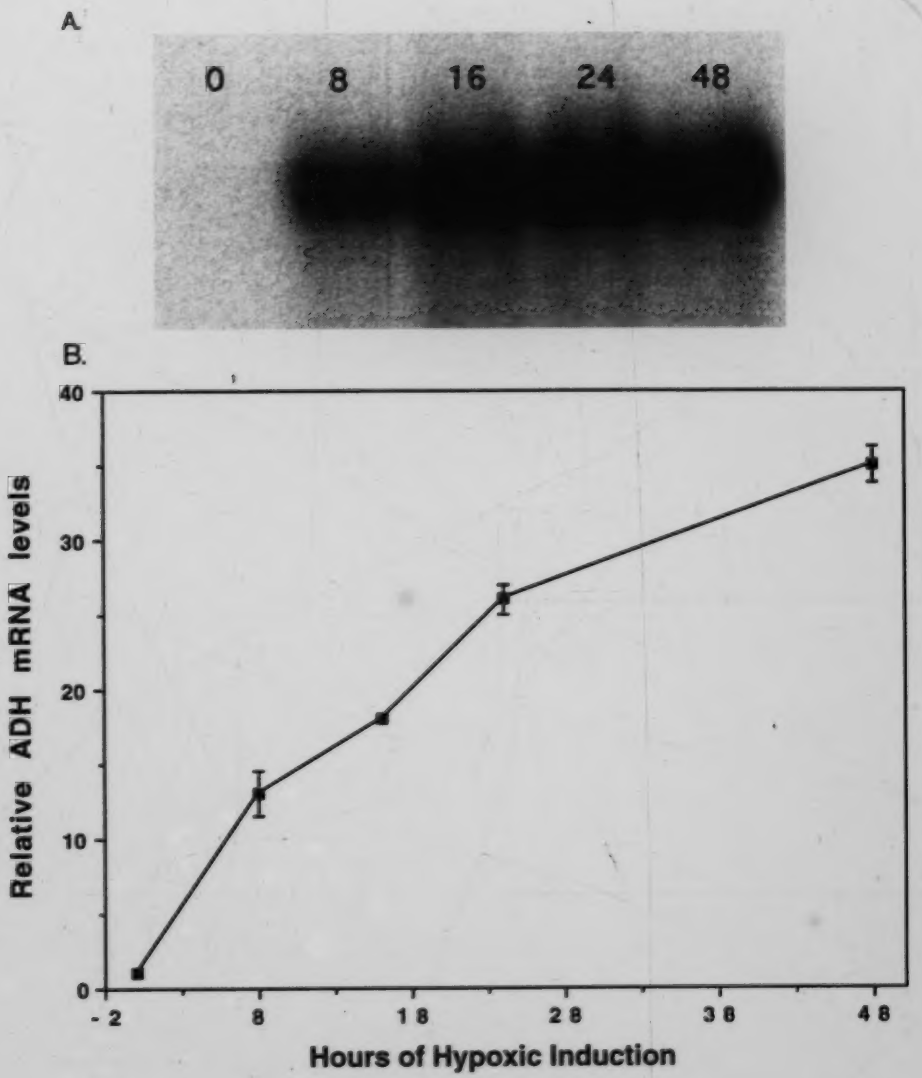


Figure 2.6. Transcriptional induction of *Adh* under hypoxic conditions.

A. A Northern blot probed with a portion of the *Arabidopsis Adh1* gene. The numbers across the top of the blot represent the hours of hypoxic exposure.

B. Relative *Adh* mRNA levels as determined by densitometric analysis. Aerobic levels were arbitrarily set to 1. Total RNA samples from three separate plants were analyzed (± standard error). Experiments were replicated three times.

A.

B.

Figure 2.7. Detection of equal loading of RNA in each lane of Northern blots.

A. Methylene blue staining of the ADH blot before probing.

B. The same ADH blot subsequently probed with an *Arabidopsis* actin fragment.

of polyA$^+$ and total RNA. The LDH mRNA (transcript of approximately 1.2 kb) rapidly accumulated during the first 16 h of hypoxia. After peaking at levels 7 to 8 fold higher than those under normoxic conditions, transcript levels declined to near aerobic levels by 48 h (Figure 2.8). Thus, hypoxic activation of both ADH and LDH occurred at the level of enzyme activity, protein, and RNA in the root tissues of *Arabidopsis* plants.

2.4 Discussion

2.4.1 Methods of anaerobic induction

Systems for creating a low oxygen environment for roots vary between research groups studying plant anaerobic stress responses. One method involving the placement of seedlings on floats in containers such that only the roots are immersed in the liquid media (Johnson et al. 1989; Dolferus et al. 1994a,b; Cobb et al. 1995). Air-tight containers housing the plants were then sealed, allowing experimental control of oxygen levels in both the air and the hydroponic growth solution by sparging with gases of known composition.

An alternative technique for anaerobic induction involves the complete submersion of seedlings in a liquid medium. Many different ways of creating both hypoxic (low oxygen) and anoxic (no oxygen) conditions stem from this basic procedure. In some experiments, seedlings have been submersed in a solution left open to the air, while in other instances, sealed containers were used to restrict air flow (Rowland and Strommer 1986; Peschke and Sachs 1994). The total submersion of seedlings in sealed containers sparged with 100% N_2 or other O_2/N_2 mixtures also has been employed to generate anoxic and hypoxic conditions, respectively (Sachs and Freeling 1978; Gerlach et al. 1982; Russell and Sachs 1992). This setup is useful for creating conditions of immediate oxygen stress.

Utilization of a variety of methods of anaerobic induction have resulted in differences in the rate at which oxygen levels decline. This variability is subsequently reflected in the nature of the anaerobic stress response. In the soil environment, the reduction of oxygen levels caused by flooding conditions occurs slowly over time. For the most part, flooding results in soil waterlogging; the complete submersion of plants occurs less frequently in dryland areas. The experimental method of anaerobic induction employed in this thesis (Section 2.2.1) involved the hypoxic treatment of the roots of *Arabidopsis* plants, while the shoots were exposed to ambient air. This system was

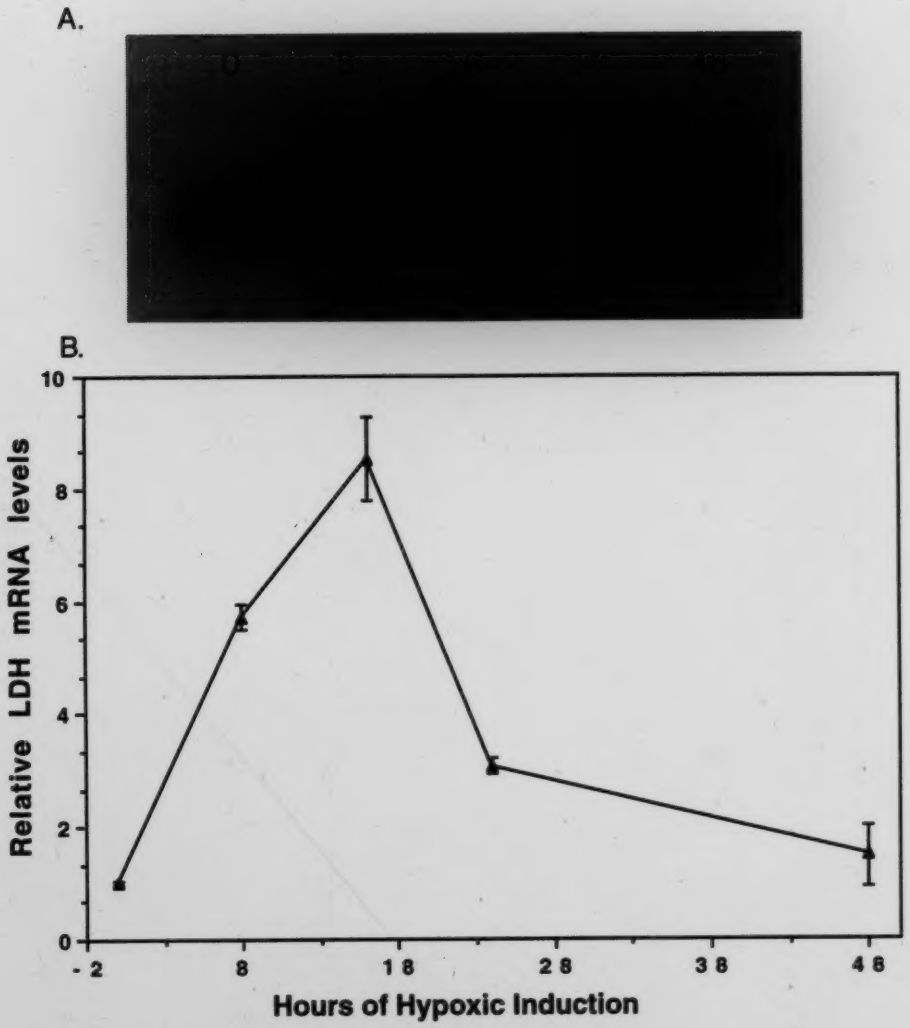


Figure 2.8. Levels of *Ldh* mRNA in roots of *Arabidopsis* grown under hypoxic conditions.

A. A Northern blot (polyA$^+$) probed with the LDH EST (expressed sequence tag) from *Arabidopsis*. The numbers across the top of the blot represent the hours of hypoxic exposure.

B. Relative expression levels of LDH as measured by densitometry. Aerobic levels were arbitrarily set to 1. PolyA$^+$ RNA samples from three plants were analyzed (± standard error). Experiments were replicated three times.

adopted for these experiments, as we felt that it most closely resembled natural flooding conditions.

2.4.2 Expression patterns of ADH and LDH under hypoxia

Activity levels of ADH increased in the roots of *Arabidopsis* plants grown under conditions of hypoxia. The observed 5 to 6 fold induction of enzyme activity resembles that of barley roots (4 to 5 fold) but is considerably lower than the 20 fold increase measured in the hypoxically pretreated roots of maize seedlings (Harberd and Edwards 1982; Mayne and Lea 1984; Johnson et al. 1989). In addition to these monocot species, similar increases in ADH activity have been reported in the roots of pea (3 to 4 fold), a dicot species. The similarity between induction in both monocots and dicots suggest a universal role for ADH activity in a plant's response to low oxygen stress.

Along with elevated levels of enzyme activity, exposure of *Arabidopsis* plants to hypoxic conditions results in larger amounts of the ADH protein and transcript. Higher levels of the ADH protein have also been found in *Arabidopsis* plantlets and various monocot species subjected to oxygen deprivation (Dolferus et al. 1985; Sachs et al. 1980; Good and Crosby 1989). Parallel increases in enzyme activity and the level of protein accumulation suggest that the higher activity levels result from larger quantities of the enzyme in roots under conditions of hypoxia. The stability of the ADH protein might also play a role in the elevation of enzyme activity. Pulse-labeling experiments using ^{35}S-methionine followed by two-dimensional polyacrylamide gels would distinguish between these two possibilities. At the RNA level, the *Arabidopsis Adh1* transcript accumulates at a slower rate than that of maize (*Arabidopsis* transcript increased up to 30 fold in 48 h versus a 50 fold increase after 7 to 10 h in maize) (Gerlach et al. 1982). Nonetheless, the increased mRNA levels suggest that the induction of the *Arabidopsis Adh1* also occurs through some form of transcriptional regulation. Dimethyl sulfate footprinting studies have delineated several regions of the *Arabidopsis Adh1* promoter thought to play a role in hypoxic inducibility, including an ARE-like element, similar to a footprinted sequence motif within the maize *Adh1* promoter (Ferl and Laughner 1989; McKendree et al. 1990; Olive et al. 1991). Perhaps the binding of a positive transcriptional regulator, the removal of a repressor protein, or a combination of these two events result in the transcriptional activation of the *Adh1* gene, ultimately leading to the observed increase of ADH activity in the root tissues of hypoxically stressed *Arabidopsis* plants.

Recently, a second *Adh* cDNA has been identified in *Arabidopsis* (Dolferus et al. 1997). Expression of *Adh2*, however, does not display the pattern of anaerobic induction seen with *Adh1*, and is transcribed at low levels in *Arabidopsis* roots (Dolferus et al. 1997a). The Northern blot analysis described in this chapter involved the use of an *Arabidopsis Adh1* fragment as a probe, and a single band was detected. Although the *Adh2* cDNA shares 63.1% sequence identity to the coding region of the *Adh1* gene, a band corresponding to the constitutively expressed *Adh2* mRNA was not detectable on these blots. Perhaps the level of *Adh2* expression is even lower than aerobic levels of *Adh1* transcription in *Arabidopsis* roots. Alternatively, if the size of the *Adh2* transcript (not yet published) was similar to that of *Adh1*, the second fainter band would be masked by a more intensely hybridizing *Adh1* band. Although *Adh2* does not contribute to elevated levels of ADH activity, the results presented in this chapter provide good evidence for the activation of *Adh1* in *Arabidopsis* roots exposed to a low oxygen environment.

LDH displays a similar pattern of induction to ADH in the roots tissues of *Arabidopsis* plants under hypoxic conditions. The 2 fold induction of LDH activity is accompanied by a concurrent increase in detectable levels of the LDH protein. The activation of LDH also occurs in maize roots (3.5 fold induction), rice seedlings (2 fold) and barley roots (20 fold) under hypoxia (Christopher and Good 1996; Ricard et al. 1991; Hoffman et al. 1986). As with ADH, the elevated activity levels may stem from increased synthesis of the LDH protein, although *in vitro* enzyme stability also may be a factor in the accumulation of the LDH protein. Tests of the *in vitro* stability of the *Arabidopsis* LDH revealed no decrease in the stability of LDH activity from hypoxically induced root samples, unlike the reduction of *in vitro* enzyme stability reported in maize (Christopher and Good 1996). Transcriptional induction of LDH in *Arabidopsis* (7 to 8 fold) resembles the elevated mRNA levels of induced barley roots (8 fold), although the LDH transcript accumulates more rapidly in *Arabidopsis* roots (16 h versus 48 h in barley) (Hondred and Hanson 1990). The brief increase of LDH transcription in *Arabidopsis* is consistent with the short term role in flooding tolerance proposed by the Davies-Roberts hypothesis (Davies et al. 1974; Roberts et al. 1984a). However, the prolonged elevation of protein and activity levels, which are also seen in barley and maize, suggest a role for LDH in a plant's response to long periods of oxygen deprivation (Hoffman et al. 1986; Good and Crosby 1989; Christopher and Good 1996).

In summary, ADH and LDH, two ANPs deemed important for flooding tolerance in other species, also are induced as part of the anaerobic stress response in the roots of *Arabidopsis thaliana*. The induction profiles of these two enzymes suggest that they could function in both short and long term responses to flooding conditions. The reactions

catalyzed by each enzyme involves the oxidation of NADH to NAD^+, a cofactor required for glycolysis. The relatively low levels of ADH and LDH under aerobic conditions indicate the minor importance of these fermentation pathways in the presence of oxygen. However, in a low oxygen environment, the activation of ADH and LDH to regenerate NAD^+ may be essential for the continuation of glycolysis.

Acknowledgements

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3 Molecular cloning of alanine aminotransferase and its expression patterns in *Arabidopsis* root tissues

3.1 Introduction

Recently, a new type of anaerobically inducible protein has been identified. This ANP, alanine aminotransferase (AlaAT), is unique in that it is involved in amino acid biosynthesis, whereas the majority of ANPs function in some aspect of glycolysis or fermentation. The AlaAT protein is a pyridoxal-5-phosphate dependent enzyme which catalyzes the reaction between pyruvate and glutamate to yield alanine and α-ketoglutarate. Since alanine has been shown to accumulate in plants under conditions of anaerobic stress, the identification of an inducible AlaAT protein may help to elucidate the pathway responsible for this elevated level of alanine production.

Under hypoxic conditions, AlaAT activity increases in the root tissues of barley and broomcorn millet (*Panicum miliaceum*) (Muench and Good 1994). The role of AlaAT in hypoxically stressed roots, however, remains to be determined. An elevation of AlaAT activity also occurs in *P. miliaceum* during leaf greening, indicating that AlaAT is light inducible in the aerial parts of the plant (Son et al. 1991). In the leaf tissues of millet, AlaAT functions in the intercellular shuttling of C_3 units (aspartate and alanine) between the mesophyll and bundle sheath cells in the NAD-malic enzyme type of C_4 photosynthesis employed by this plant (Hatch 1987; Son et al. 1991).

AlaAT has not been studied in any dicot species to date. Preliminary experiments with pea roots indicated that AlaAT activity was not inducible under hypoxic conditions (Good and Muench, unpublished results). Expression patterns of AlaAT were examined in *Arabidopsis* roots under hypoxia to determine if the lack of induction observed in pea extended to other dicot species. Since the initial sets of enzyme assays demonstrated the hypoxic induction of AlaAT activity, levels of enzyme activity, protein, and mRNA were measured under both aerobic and hypoxic conditions to determine if AlaAT functioned as an ANP in *Arabidopsis*. The *AlaAT* gene from *Arabidopsis* was thus cloned to permit subsequent analysis of the promoter region. The isolation of the promoter will allow comparisons to be made between the regulation of *AlaAT* and other anaerobically inducible genes in *Arabidopsis*.

3.2 Materials and Methods

3.2.1 Assay of AlaAT activity

Arabidopsis plants were grown, induced, and harvested for enzyme assays as described previously (Sections 2.2.1 - 2.2.2). Activity of AlaAT was measured spectrophotometrically at 340 nm, based on the oxidation of NADH. Activity levels were assayed by a two step reaction (Appendix 5.2.3). The conversion of alanine to pyruvate was coupled to the oxidation of NADH by LDH, which produces a decrease in absorbance at 340 nm (Good and Crosby 1989). The reaction buffer consisted of 0.1 M Tris-HCl (pH 8.0), 0.1 mM NADH, 10 mM α - ketoglutaric acid, and 10 units of LDH (Sigma L2375) in a final volume of 1 ml. Initial absorbance readings were taken of this assay buffer, mixed with 50 µl of root extract, to assess background levels of NADH oxidation. The AlaAT reaction was initiated by the addition of 20 mM alanine to the assay buffer/root extract mixture. The absorbance of this reaction was monitored for 2 min. One international unit (IU) of AlaAT activity was defined as the amount of enzyme which catalyzed the formation of 1.0 µmol of product per min at 23°C. Additional enzyme assays were performed, as described above, with 20 µg/ml to 45 µg/ml pyridoxal 5 - phosphate included in the reaction buffer (Hatch 1973). Total protein concentrations were determined using the Bradford assay, as previously described (Section 2.2.5).

3.2.2 Protein gel electrophoresis

Protein samples were run in both non-denaturing and denaturing polyacrylamide gels, as described previously (Sections 2.2.6 - 2.2.7). In a subset of native gels, samples were mixed with 75 µg/ml pyridoxal 5-phosphate and were incubated at room temperature for 30 min, prior to loading. These samples then were mixed with loading dye (65 mM Tris-HCl [pH 6.5], 37.5% glycerol, and 0.1% [w/v] bromophenol blue) and were loaded onto native gels, which were run under the conditions discussed in Section 2.2.6.

3.2.3 Western blotting and antibody staining

Proteins on both denaturing and non-denaturing gels were transferred to nitrocellulose membranes by electroblotting, as described in Section 2.2.10. Detection of the AlaAT protein on these blots was performed using both the alkaline phosphatase and streptavidin-horseradish peroxidase methods (Section 2.2.10). In each case, the AlaAT primary antibody originally made from the purified barley protein was diluted

1:3000, with the remaining conditions identical to those described earlier (Muench and Good 1994). Protein densitometry was carried as described in Section 2.2.13.

3.2.4 RNA preparation and Northern blots

Total RNA and mRNA preparations, RNA gel electrophoresis, and Northern blotting procedures were performed as described in Section 2.2.11.

3.2.5 Phage, bacteria and DNA manipulations

All procedures involving the growth and handling of bacteria and λ phage were carried out as outlined by Sambrook et al. (1989). More detailed accounts of the modified protocols used for λ and plasmid DNA isolations, plant DNA extractions, and Southern blotting (capillary method) are provided in sections 5.3 through 5.6 of the Appendix.

3.2.6 Genomic library screening

A λ phage library (vector: λ FIX) made from whole plant genomic DNA from the Landsberg strain of *Arabidopsis thaliana* was obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University. Approximately 10,000 plaques were plated on each of 10 - 150 mm plates (100,000 plaques screened overall). Duplicate plaque lifts were performed on each plate using nylon membranes (MAGNA nylon, Micron Separates Inc.). A partial AlaAT cDNA clone (pB8XP), in the plasmid vector pZL1, also obtained from Ohio State University, was radioactively labeled with ^{32}P - dCTP, using the random priming method (Sambrook et al. 1989). The plaque lifts were hybridized at 65°C for 14 - 16 h in 50 ml of hybridization solution (5X SSPE, 1X Denhardt's solution, 0.2% SDS, and 100 µg/ml salmon sperm DNA). The lifts were subsequently washed twice at room temperature for 10 min in 2X SSC (0.3 M sodium chloride and 30 mM sodium citrate), once at 65°C for 30 min in 2X SSC, and once at 65°C for 30 min in 0.7X SSC, 0.1% SDS. The membranes were exposed to X-ray film for 24 to 48 h at -80°C. Potentially positive phage clones subsequently were isolated, replated, and subjected to two additional rounds of screening. DNA was isolated from the positive clones, and a restriction map was prepared of λ4-1-1.

3.2.7 Sequence analysis

Arabidopsis genomic DNA fragments from one positive λ clone (λ4-1-1) was subcloned into pT7T3 18U (Pharmacia). The Exo III/mung bean deletion kit

(Stratagene) was used to make nested deletions from one end of the insert. The remainder of the sequence was obtained by designing primers based on the AlaAT cDNA sequence. Double stranded sequencing of both strands of the genomic subclones were performed using the T7 sequencing kit (Pharmacia), based on the method of Sanger et al. (1977). Additional sequence was obtained by PCR-based automated sequencing using the ABI 377 automated sequencer.

3.3 Results

3.3.1 Induction of AlaAT activity by hypoxia

Two interesting results were obtained from the AlaAT enzyme assay experiments. The first result was that AlaAT activity under aerobic conditions (approximately 7.5 IU per mg total protein), was noticeably higher than aerobic levels of LDH activity (approximately 0.1 IU per mg total protein) and, to a lesser extent, ADH activity (approximately 5.07 IU per mg total protein). The second result indicated AlaAT activity levels slowly increased by about 2 fold relative to aerobic levels over a 96 h hypoxic treatment (Figure 3.1). A similar induction pattern was detected in the roots of R001 (*Adh*$^-$) mutants. Since no significant differences in AlaAT activity levels were found between the wild-type and mutant strains, further analysis of AlaAT expression was carried out using only wild-type plants.

3.3.2 Immunological detection of the AlaAT protein

Relative amounts of the AlaAT protein were measured under both normal and low oxygen conditions to determine if a correlation existed between enzyme activity and the amount of protein in roots of *Arabidopsis*. Immunological staining of Western blots using the barley AlaAT antibody (Muench and Good 1994) revealed a parallel increase in the amount of protein (2 fold) in the root tissues, following a 96 h hypoxic treatment. A comparison of pre-immune and antibody stained blots indicated that the barley antibody was detecting the *Arabidopsis* AlaAT protein, with very few background bands. Accumulation of a single protein band was detected on blots made from both denaturing and native gels. However, this protein band exhibited slightly different migration patterns on these two types of gels (Figure 3.2, 3.3). The immunological detection of AlaAT from protein extracts electrophoresed on native gels revealed a noticeable difference between aerobic and induced samples (Figure 3.2). On immunoblots from SDS gels, the denatured AlaAT protein consistently migrated to the same location, within the

50 kD range, regardless of the type of sample (aerobic versus hypoxic) used (Figure 3.3). The native AlaAT polypeptide obtained from aerobic root extracts migrated slightly more rapidly than that present in hypoxically stressed root samples. This band shift appeared in all native immunoblots and could not be attributed to the uneven distribution of heat across the gel or to any other variable with respect to the gel electrophoresis conditions.

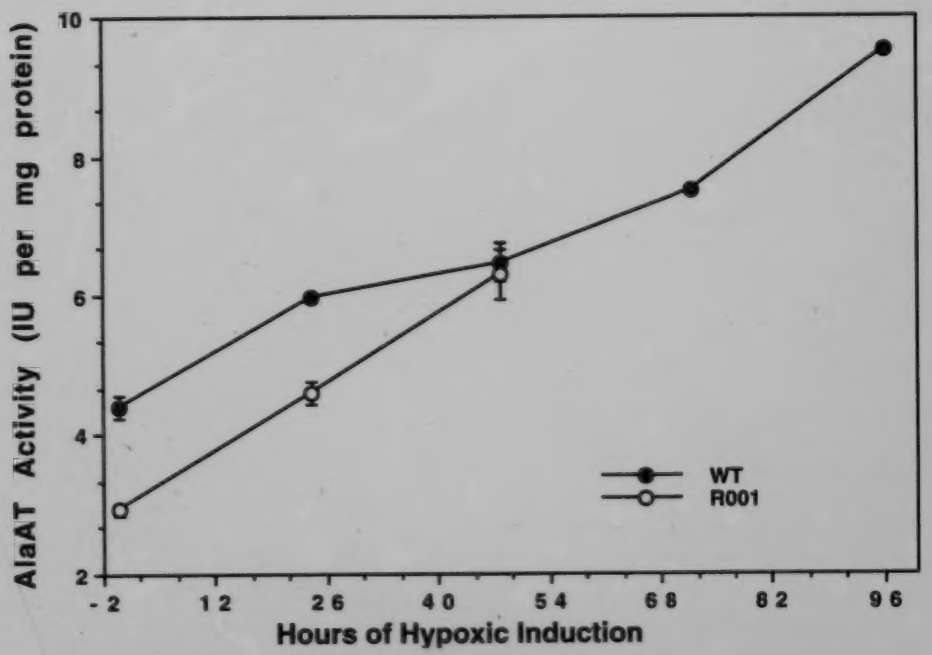
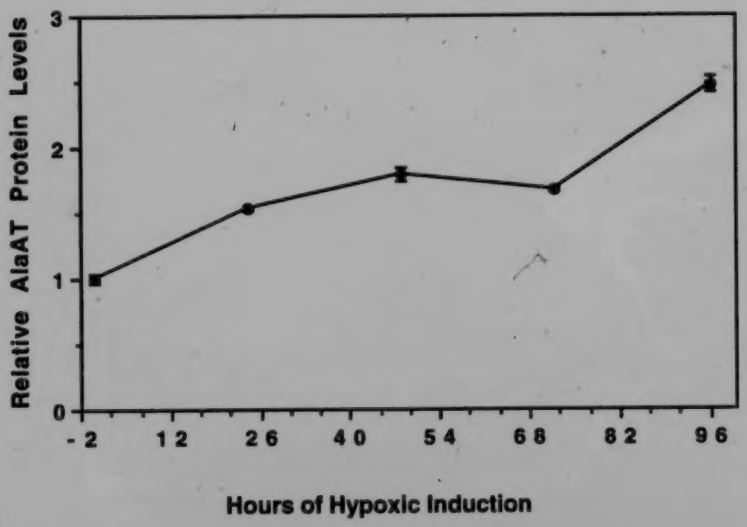


Figure 3.1. AlaAT activity in wild-type (WT) and R001 (ADH^-) *Arabidopsis* roots under hypoxia. Activity levels were determined by spectrophotometric enzyme assays. The value at each time point represents the mean of the data obtained from a single experiment (n = 3; ± standard error). These experiments were replicated three times.

In an attempt to uncover the nature of this band shift, an excess of pyridoxal-5-phosphate (PLP) was incubated with both uninduced and induced root extracts, prior to loading on a native gel. PLP binds to many plant aminotransferases, and its binding has been shown to increase enzyme activity (Hatch 1973; Hondred et al. 1985). If PLP played a role in the altered protein mobility, the incubation of excess PLP with both aerobic and hypoxic root extracts should saturate all PLP binding sites and eliminate the band shift present on native gels. However, the addition of PLP had no effect on native protein migration patterns (data not shown).

A.



B.

Figure 3.2. Levels of the AlaAT protein in roots of *Arabidopsis* under hypoxic conditions.

A. Protein concentrations were measured by densitometry of native and SDS immunoblots. Aerobic levels were arbitrarily set to 1. The value at each time point represents the mean of the data from three separate plants (± standard error). Experiments were replicated three times.

B. Native immunoblot probed with the barley AlaAT polyclonal antibody. The numbers across the top of the blot indicate the hours of hypoxic induction.

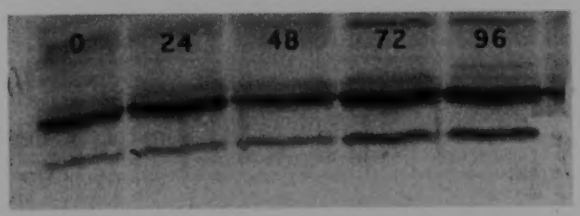


Figure 3.3. An SDS immunoblot probed with the barley AlaAT antibody, illustrating the absence of a band shift when samples were denatured The AlaAT protein is the upper band. The numbers across the top of the blot represent the hours of hypoxic exposure.

Since the band shift was present only between the 0 and 24 h time points of hypoxic treatment, the event causing this altered protein mobility occurs during this period. If the change in the native AlaAT protein occurred gradually over time, a transitionary phase may exist in which two forms of the AlaAT protein would be present in root extracts. Tissue extracts were prepared from the roots of plants that had been hypoxically induced for 0, 4, 8, 16, and 24 h to determine if this transition period does produce 2 protein bands. Enzyme activity levels were measured by spectrophotometric assays to ensure that an active AlaAT protein was present in each root extract, prior to loading on non-denaturing polyacrylamide gels. From antibody stained Western blots, prepared with these samples, only one protein band was detected in each lane. The band shift was not visible. The factor responsible for generating this band shift remains unknown.

3.3.3 Accumulation of the AlaAT transcript under hypoxia

Hypoxic induction of AlaAT mRNA preceded the increase in enzyme activity and the amount of protein. Total RNA samples prepared from uninduced and induced root tissues were electrophoresed on agarose gels, blotted, and subsequently probed with a 1.3 kb PstI/HindIII fragment from plasmid pB8XP. This plasmid contains a partial cDNA corresponding to the *Arabidopsis* AlaAT. This probe detected a band, approximately 1.4 kb in size, that displayed a pattern consistent with hypoxic induction. AlaAT transcript levels rapidly increased during the first 8 h of exposure to hypoxia, peaking at 8 to 10 times the aerobic mRNA levels after 24 h (Figure 3.4). Transcript levels had noticeably decreased by 48 h of hypoxia 2 to 3 fold aerobic levels. Equal loading of RNA samples was monitored by methylene blue staining and probing the blots with the *Arabidopsis* actin.

3.3.4 Molecular analysis of the AlaAT gene

Approximately 100,000 plaques were screened from a λ FIX *Arabidopsis* genomic library using the 1.3 kb PstI/HindIII fragment (partial *AlaAT* cDNA) from pB8XP as a probe. From this screen, 8 putative *AlaAT* λ clones were isolated. DNA was extracted from all 8 phage clones. Southern blot analysis of the clones indicated that each strongly hybridized to the partial *AlaAT* cDNA (data not shown). One of the 8 λ clones (λ4-1-1) was analyzed further, and a restriction map was prepared.

The plant genomic DNA insert contained within the phage clone λ4-1-1 was approximately 18 kb in size (Figure 3.5). From this insert of Arabidopsis DNA, three EcoRI fragments (0.8 kb, 1.8 kb, and 3.0 kb), along with a 3.1 kb XbaI/AvaI fragment

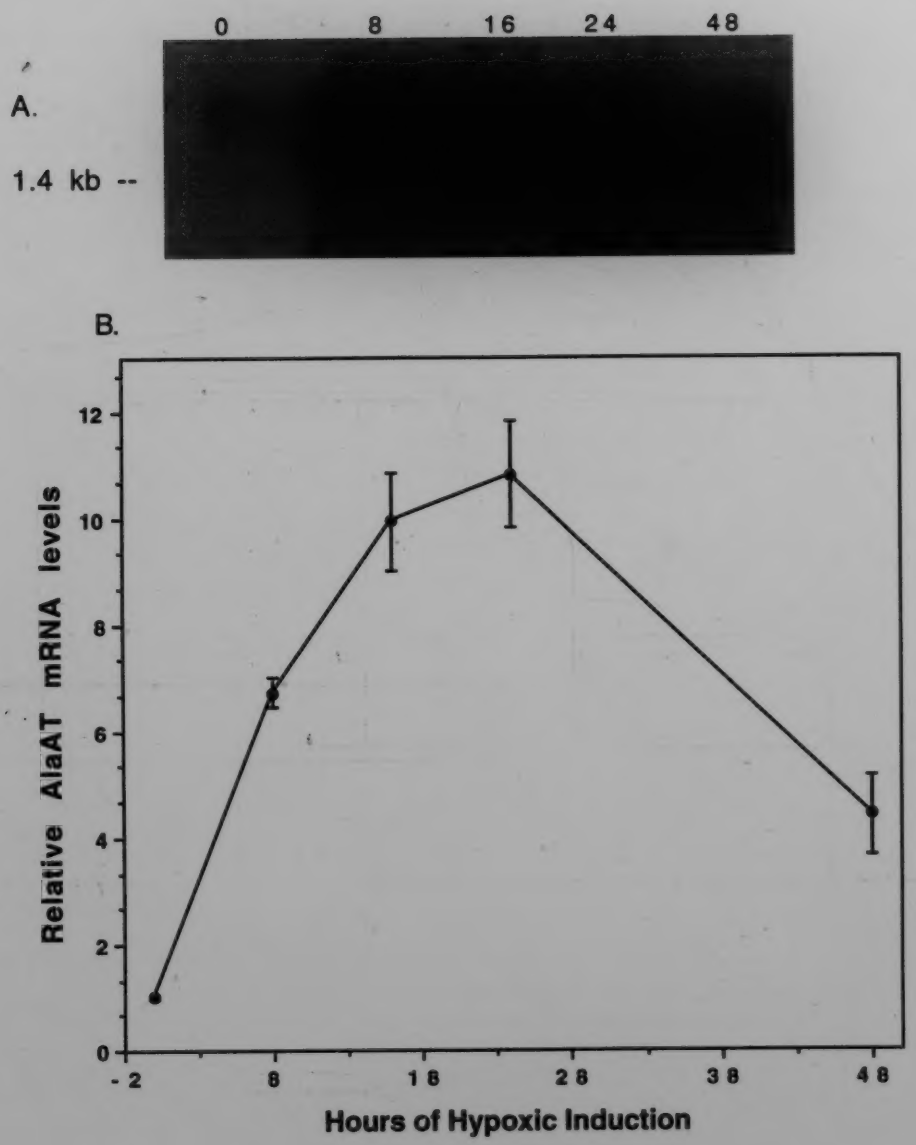


Figure 3.4. Levels of *AlaAT* mRNA in roots of *Arabidopsis* grown under hypoxic conditions.

A. A Northern blot (total RNA) probed with the partial *AlaAT* cDNA fragment from pB8XP. The numbers across the top of the blot are indicative of the length of the hypoxic treatments. The blot was overexposed such that the 0 h band could be seen.

B. Relative *AlaAT* transcript levels as determined by densitometric analysis. Aerobic levels were arbitrarily set to 1. Total RNA samples from three separate plants were analyzed (± standard error). Experiments were replicated three times.

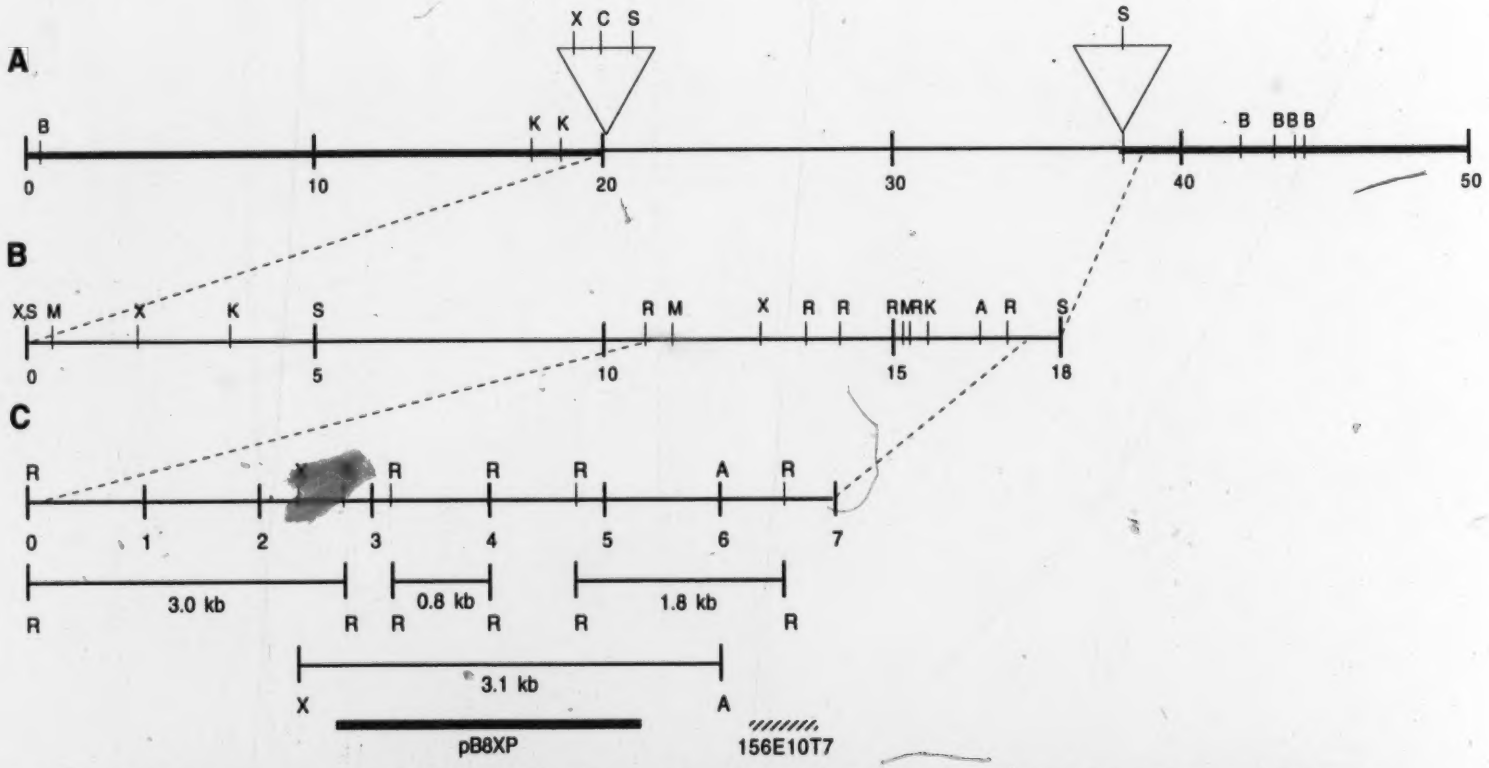


Figure 3.5. Restriction maps of the *Arabidopsis AlaAT* genomic λ clone (λ4-1-1). A) A map of the λ vector, with the multiple cloning sites shown on the inverted triangles. The thick bars represent the left and right λ arms. The plant DNA insert is denoted by a thin line. B) A detailed map of the 18 kb plant DNA insert. All of the R and A sites have not been marked on this map, as the locations of all these sites could not be unambiguously determined. C) An enlargement of the region from which fragments were subcloned. The relative locations of the various subclones (Section 3.3.4) are shown below, along with the partial cDNA (pB8XP and 156E10T7). Numbers indicate distances (kb) from the left end of each DNA fragment. Restriction enzyme abbreviations: B, BglII; K, KpnI; X, XbaI; C, SacI; S, SalI; M, SmaI; R, EcoRI; A, AvaI.

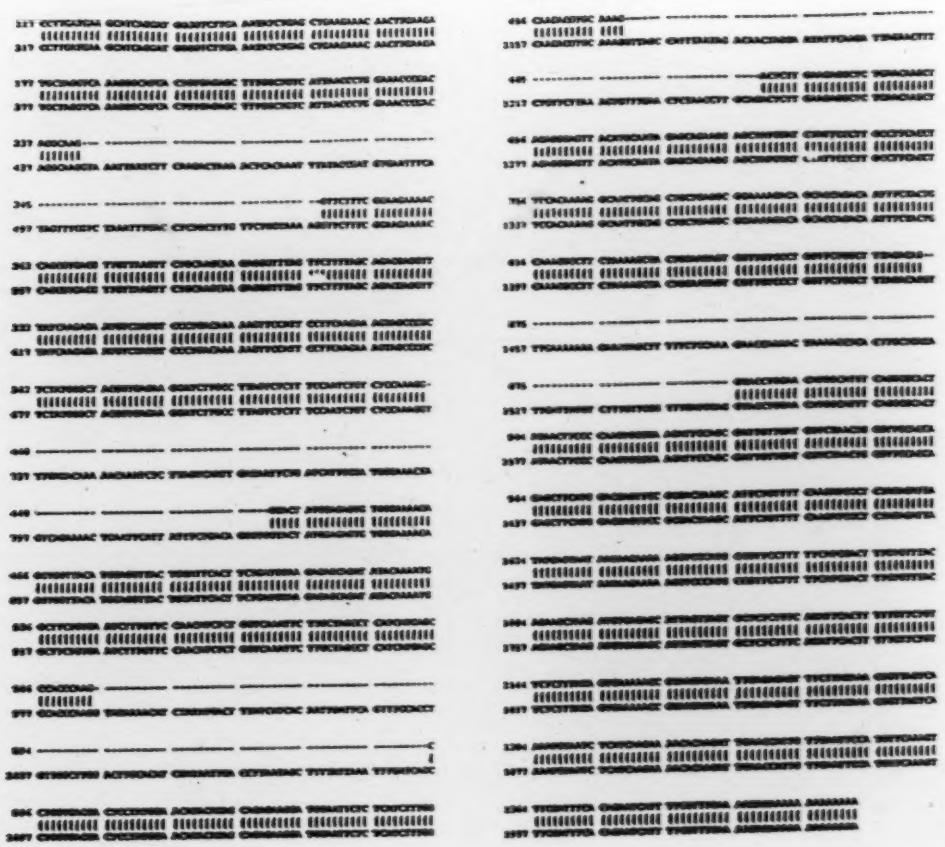
were subcloned into the plasmid vector pT7T3 18U for sequencing. The 3.0 kb EcoRI fragment contained the 3' end of the cDNA and substantial downstream sequences. The 800 bp EcoRI subclone was localized to the middle of the partial cDNA, whereas the 1.8 kb EcoRI fragment contained part of the 5' region. The 3.1 kb XbaI/AvaI subclone overlapped with the entire 0.8 kb EcoRI fragment, along with portions of the 1.8 kb and 3.0 kb EcoRI fragments.

Comparisons between the genomic and partial cDNA sequences indicated the presence of at least 5 introns, ranging in size from 80 bp to 128 bp, and a minimum of 8 exons (Figure 3.6). For the most part, the intron-exon boundary sequences conformed to the *Arabidopsis* splice site consensus (AG/GT). Closer examination of the partial cDNA sequence revealed an in-frame stop codon (TAG) at base 571, indicating that the remaining sequence comprised the 3' untranslated region of the gene. The translatable region, based on the 570 bases upstream of this termination codon, would have produced a 190 amino acid protein, with an approximate molecular weight of 20.9 kD. The size of the AlaAT protein, as estimated from denaturing polyacrylamide gel electrophoresis, was approximately 50 kD. Taken together, these results indicated that the partial AlaAT cDNA contained within plasmid pB8XP lacked the 5' end of the gene.

A BLAST search on the internet, using the full length maize AlaAT cDNA sequence (predicted from the genomic sequence), identified a second partial AlaAT cDNA clone from *Arabidopsis* with homology to the 5' end of the maize cDNA. This cDNA clone (156E10T7) was isolated by a research group partially sequencing random *Arabidopsis* cDNA clones (Newman et al 1994). Comparisons between the 156E10T7 sequence and genomic sequences upstream of the pB8XP sequence revealed segments of sequence identity, supporting the notion that the 156E10T7 clone contained a portion of the 5' end of the *Arabidopsis AlaAT* gene. Additional primers were designed from the 156E10T7 sequence to obtain the remaining 5' sequence from the 1.8 kb EcoRI subclone. However, the 1.8 kb EcoRI fragment also was found to be missing the 5' region of the gene. The sequence of the 1.8 kb EcoRI fragment ends approximately 150 bases downstream of the 5' end delineated by the 156E10T7 clone. The 156E10T7 clone itself does not contain the transcriptional start site for the *Arabidopsis AlaAT* gene, although sequence comparisons with both the maize and barley AlaAT cDNAs indicate that this partial cDNA lies well within the 5' region of the *AlaAT* gene.

Southern blot analysis of *Arabidopsis* genomic DNA using the 1.3 kb fragment of pB8XP as a probe, produced a simple pattern of a few hybridizing bands, suggesting that *Arabidopsis* contains only one *AlaAT* gene (Figure 3.7). The numbers of restriction sites

A.



B.

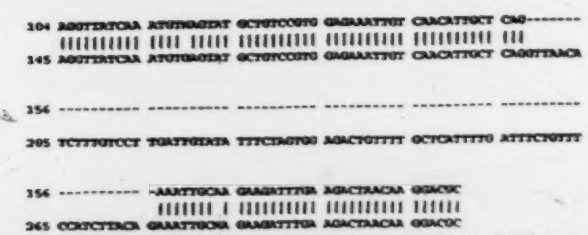


Figure 3.6. A comparison of the genomic and cDNA sequences of the *Arabidopsis AlaAT*, indicating the intron-exon boundaries.

A. A comparison of the pB8XP sequence (top) with the genomic sequence (bottom).

B. The alignment between the 156E10T7 sequence (top) and the genomic sequence (bottom).

of the various enzymes determined from the Southern blot match the sites present in the genomic sequence. Single *AlaAT* genes also have been identified in barley and maize

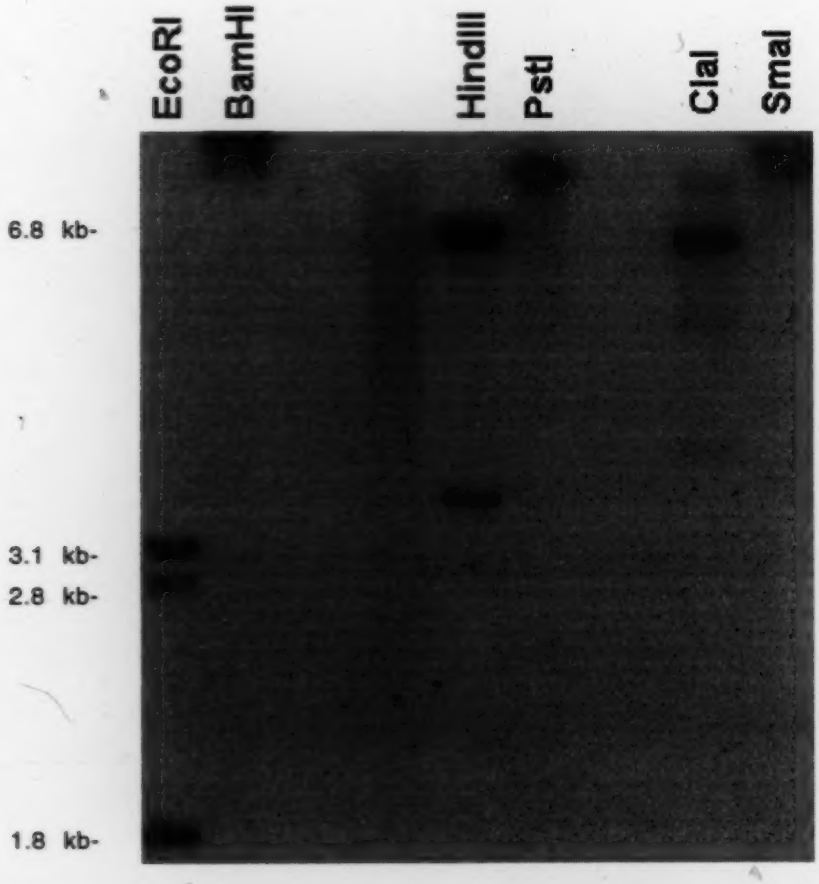


Figure 3.7. A Southern blot of *Arabidopsis* genomic DNA probed with the partial cDNA fragment from pB8XP.

(Muench and Good 1994; Muench et al. submitted). The detection of a single band on both Western and Northern blots supports the idea that *Arabidopsis* has only one copy of the *AlaAT* gene. Based on these results, along with the identification of regions of sequence homology, it is likely that the partial cDNA clones 156E10T7 and pB8XP comprise different regions of the same *AlaAT* gene.

The *Arabidopsis AlaAT* sequence has also been compared to *AlaATs* identified in other plant species. Homology was found between *Arabidopsis* and barley at both the

protein and DNA levels (Figure 3.8, 3.9). The pB8XP sequence was provided by R. Dolferus (C.S.I.R.O., Australia). A partial 156E107 sequence was obtained from the BLAST search. At the DNA level, the larger *Arabidopsis* cDNA (pB8XP) shared approximately 60% sequence identity with the 1.7 kb barley cDNA, isolated by Muench and Good (1994). Comparisons between the AlaAT cDNA sequences from both *Arabidopsis* (pB8XP) and maize (Muench et al. submitted) revealed 62.1% sequence identity (Figure 3.10). The results of these sequence comparisons indicate that *Arabidopsis* shares homology with two monocot *AlaAT* sequences.

3.4 Discussion

Upon exposure to low oxygen conditions, AlaAT activity levels increase in roots of *Arabidopsis* plants. These elevated enzyme activities suggest that AlaAT may have an important role in the survival of hypoxia. Increases in AlaAT activity also have been reported in root tissues of many cereal crop plants, including barley, wheat, rye, and maize (Good and Crosby 1989; Muench and Good 1994; Muench et al. submitted). Hypoxic induction of enzyme activity correlates well with the elevated levels of alanine found in plant roots under anaerobic conditions (Smith and ap Rees 1979; Hoffman et al. 1986). In a reduced oxygen environment, much of the alanine produced remains within the roots. The fate of alanine differs from that of ethanol, since most of the ethanol produced diffuses into the external medium (Hoffman et al. 1986). The accumulation of alanine may benefit the plant by sequestering carbon and nitrogen molecules within the cell for the synthesis of other amino acids and proteins upon the return of aerobic conditions. In addition to this function, the presence of free amino acids within the vacuolar compartments of plant cells, where the excess alanine is thought to be stored, may have a role in cytoplasmic homeostasis (Sakano and Tazawa 1984). The correlation between increases in alanine production and AlaAT activity strongly suggests that AlaAT is at least partially responsible for the elevated synthesis of alanine under hypoxic conditions.

A parallel increase in the amount of the AlaAT protein also occurred in the roots of hypoxically stressed *Arabidopsis* plants. Analysis of protein accumulation by native immunoblots revealed an unusual difference in electrophoretic mobilities between the uninduced and induced forms of AlaAT. This change in migration of the native protein was postulated to be the result of a cofactor binding the enzyme under hypoxic conditions. Pyridoxal-5-phosphate (PLP) is an important component of many plant aminotransferases and was originally thought to be a likely candidate, however, the

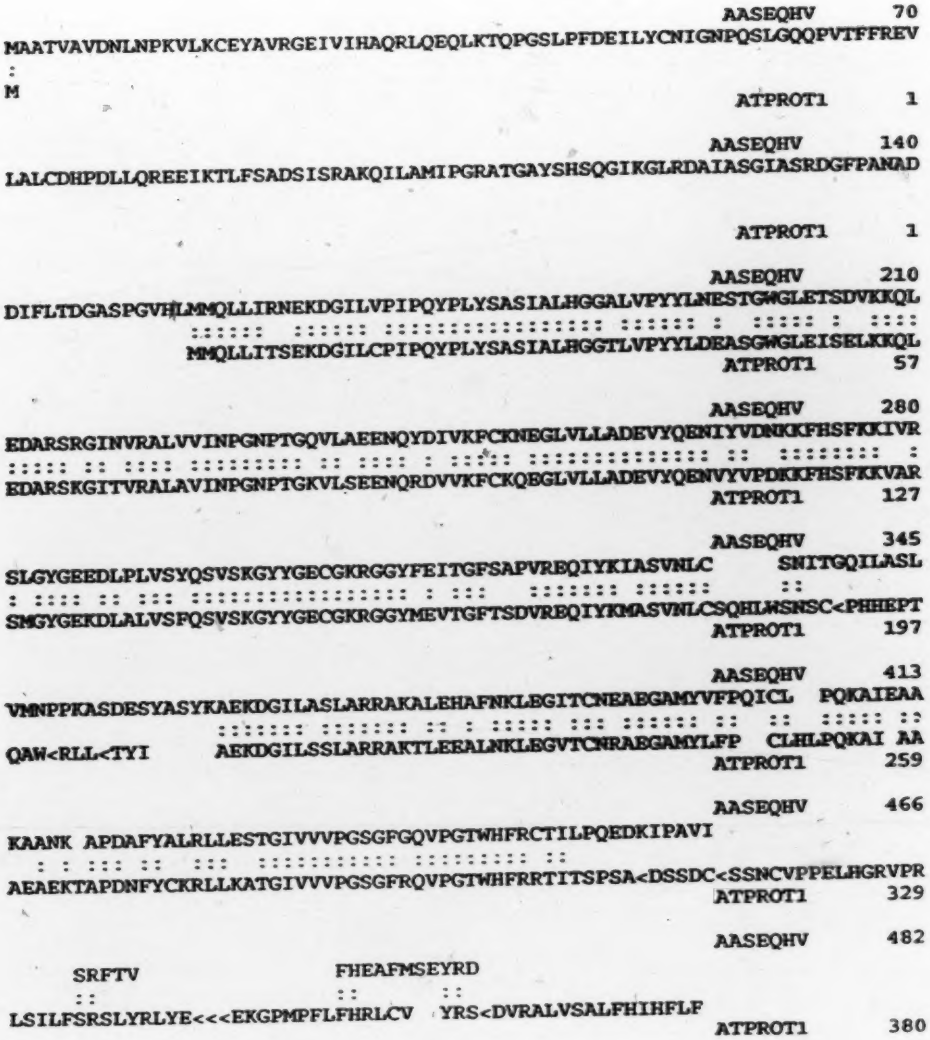
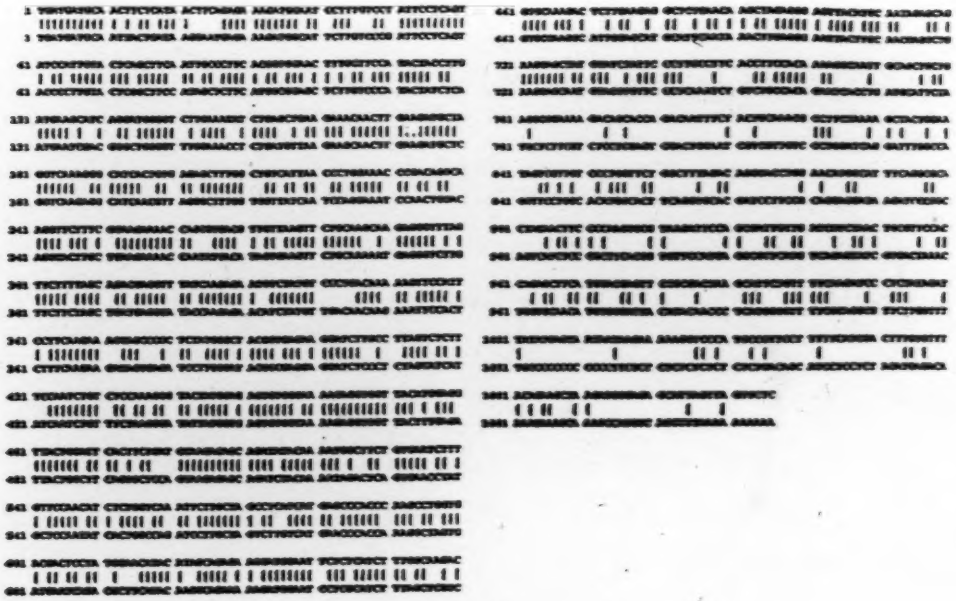


Figure 3.8. The alignment between the predicted amino acid sequences from the barley AlaAT (top) and *Arabidopsis* (pB8XP - bottom) cDNAs. AASEQHV, barley amino acid sequence; ATPROT1, pB8XP amino acid sequence.

A.



B.

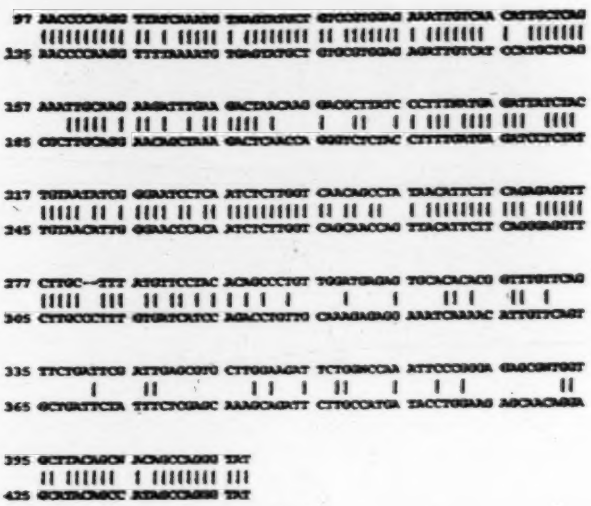
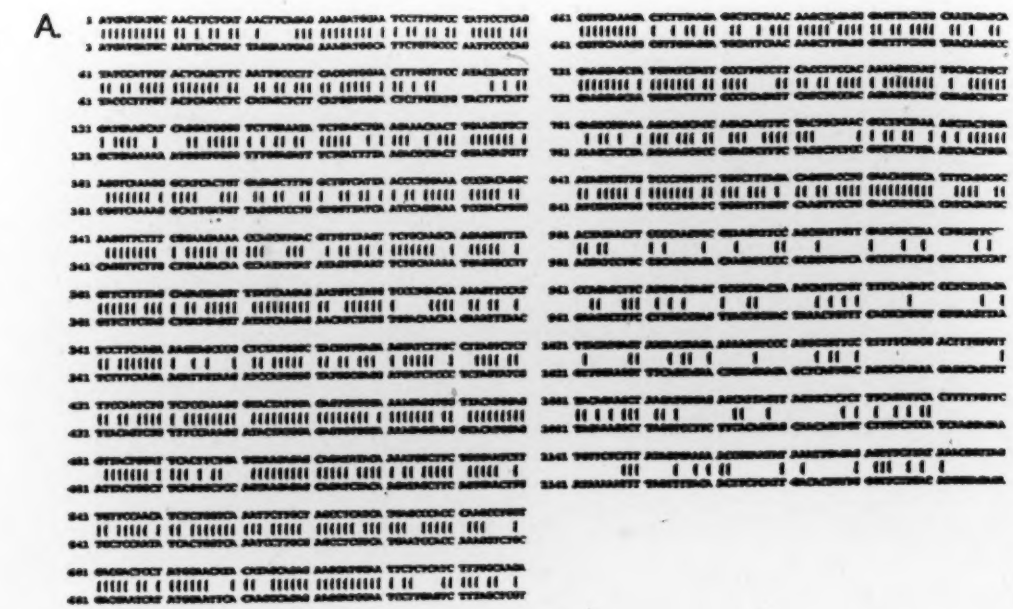


Figure 3.9. A homology comparison between the *Arabidopsis* and barley *AlaAT* cDNAs.

A. A DNA sequence alignment between pB8XP (top) and the barley cDNA (bottom) starting at base 555 of the barley sequence.

B. A comparison between the 156E10T7 sequence (top) and the barley cDNA sequence (bottom).



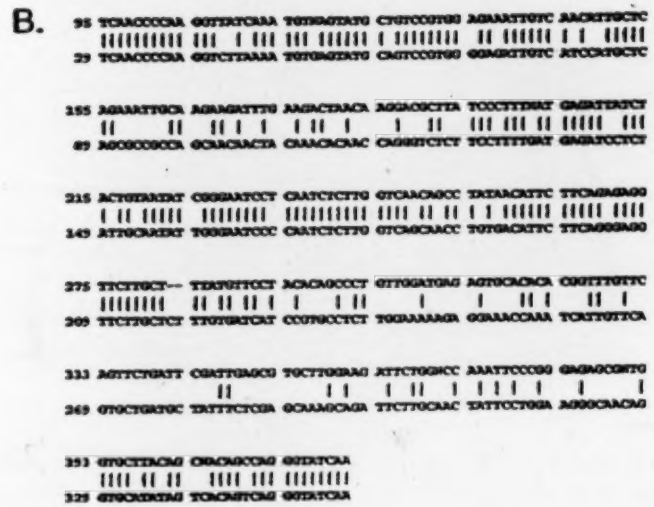


Figure 3.10. DNA sequence alignments between the *Arabidopsis AlaAT* cDNAs and the predicted maize cDNA.

A. A comparison between pB8XP (top) and maize (bottom), starting from base 460 of the maize sequence.

B. The alignment of 156E10T7 (top) and the maize cDNA (bottom).

incubation of root extracts with PLP had no effect on migration patterns (Hatch 1973). The absence of an effect of PLP incubation with roots extracts on the migration patterns of the native AlaAT protein does not rule out a possible influence of PLP on AlaAT activity. PLP may still interact with AlaAT under normal aerobic conditions and maintain this association under periods of oxygen stress. The band shift may result from the interaction of an unknown cofactor that specifically associates with AlaAT under hypoxic conditions.

Elevated levels of enzyme activity and the amount of protein are preceded by an increase in the level of AlaAT mRNA present in roots of *Arabidopsis* grown under hypoxia. Accumulation of the AlaAT transcript also has been detected in barley (4 fold higher than aerobic levels) and maize (32 fold) roots exposed to a limited oxygen environment (Muench and Good 1994; Muench et al submitted). Induction in all three species follows a similar pattern consisting of a rapid increase of AlaAT mRNA, after which the transcript levels decline but still remain above aerobic levels. Similar patterns of transcript accumulation have also been observed with the *Adh* and *Ldh* genes in maize (Gerlach et al. 1982; Dennis et al. 1985; Christopher and Good 1996). In the case of the *Arabidopsis* AlaAT, the initial increase in the amount of mRNA occurs well before the corresponding increases in the amount of protein and the level of enzyme activity. These results suggest that the translation of the AlaAT mRNA lags behind transcription. However, as AlaAT mRNA levels start to decline after 24 h of hypoxic treatment, the amount of protein and level of enzyme activity continue to increase. This discrepancy between transcript and protein levels may reflect the high stability of the AlaAT enzyme under hypoxic conditions. Taken together, these results indicate that the hypoxic inducibility of AlaAT in *Arabidopsis* may be regulated at both the transcriptional and post-translational levels.

The *Arabidopsis* AlaAT shares significant amounts of sequence homology with *AlaAT* genes from cereal crop plants. The DNA sequence of the *Arabidopsis AlaAT* more closely resembles that of maize (62.1 %) than barley (60 %). Unfortunately, sequence data is presently unavailable for any dicot *AlaAT* genes, preventing a direct comparison between species within this subdivision of plants. The promoter region, along with the 5' end of the gene, still have to be subcloned and sequenced. Primer extension analysis can subsequently be performed to determine the exact location of the transcriptional start site. The promoter region also can be examined for specific sequence elements found in the promoters of other ANPs that are thought to be involved in the regulation of hypoxic gene expression.

In summary, the gene encoding a unique type of anaerobic protein involved in amino acid biosynthesis has been identified in *Arabidopsis*. Studies of AlaAT expression indicated that hypoxia induces elevated levels of enzyme activity, protein, and mRNA. The pattern of AlaAT expression under hypoxic conditions is similar to that of other species, suggesting that AlaAT has a role in the anaerobic stress response in the roots of *Arabidopsis thaliana*.

Acknowledgements

I would like to thank Rudy Dolferus for providing the complete sequence of the AlaAT insert in pB8XP. I would also like to thank James Maclagan for his technical assistance with the automated sequencing.

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4 General Discussion

The emergence of *Arabidopsis thaliana* as a model organism for higher plant species has prompted many research efforts in a wide variety of areas, including anaerobic stress. Characterization of the role of ADH in the response of *Arabidopsis* to limited oxygen availability through mutational analysis, gene isolation, and promoter dissection studies have set the stage for the examination of the functional significance of other ANPs in *Arabidopsis*. This thesis project was undertaken in 1993 to further the knowledge of the flooding response of *Arabidopsis* by exploring the expression patterns of ADH, LDH, and AlaAT in root tissues subjected to hypoxia.

4.1 Patterns of ADH, LDH, and AlaAT Expression Under Hypoxia

Levels of ADH and LDH expression increase in *Arabidopsis* roots upon exposure to hypoxia. Induction of ADH and LDH is manifested at the levels of enzyme activity, amounts of protein, and levels of transcription (Chapter 2). Despite their similar expression patterns, the overall levels of LDH activity under both aerobic and hypoxic conditions are lower than those of ADH activity. These differences in activity levels may reflect the relative importance of the two enzymes under hypoxia. High levels of ADH and ethanol production may be more beneficial to the plant under conditions of limited oxygen availability than the synthesis of lactate by LDH. Both reactions result in the regeneration of NAD^+ for continued glycolysis. Although metabolite levels have yet to be measured in hypoxically stressed *Arabidopsis* roots, ethanol fermentation appears to be a universal response to anaerobic conditions, observed in both flood tolerant and sensitive species (Cobb and Kennedy 1987; Drew 1996). Ethanol has been shown to diffuse out of barley roots under hypoxia, while exerting no toxic effect at the cellular level (Hoffman et al. 1986). Lactate is secreted from roots grown under conditions of hypoxia, although to a lesser degree than ethanol. In some members of the halophytic genus *Limonium*, high levels of lactate synthesis were sustained for several hours in hypoxically acclimated root tissues subsequently exposed to anoxia (Rivoal and Hanson 1993). A large portion of the lactate produced was secreted into the external medium and did not cause acidification of the cytoplasm (Rivoal and Hanson 1993). However, in other dryland plant species, lactate can accumulate within cells and has been associated with cytoplasmic acidosis (Hoffman et al. 1986; Roberts et al. 1984a,b). Lactate production occurs at low levels in aquatic species like wild rice (*Zizania palustris* L.) (Muench et al. 1993). The magnitude of the hypoxic induction of LDH activity in wild

rice is less than that seen in dryland cereals like maize (Christopher and Good 1996). Under anaerobic conditions, a cytosolic alkalinization is observed in cells of flood tolerant species like rice and barnyard grass (Menegus et al. 1989; 1991). The absence of cytoplasmic acidosis in flood tolerant plant species may partially be attributed to low levels of lactate production. Based on these results, higher levels of ethanol fermentation by ADH, relative to lactate production by LDH, may be the better pathway for NAD^+ regeneration under conditions of oxygen stress.

Like ADH and LDH, AlaAT expression is hypoxically inducible in the roots of *Arabidopsis*. Transcript levels of AlaAT rapidly increase under conditions of low oxygen availability, followed by a corresponding increase in the amount of protein and the level of enzyme activity (Chapter 3). The comparatively high aerobic levels of AlaAT, relative to those of ADH and LDH, may reflect the importance of AlaAT in alanine production under both normal and reduced oxygen conditions. Similar increases in AlaAT enzyme activity have been reported in the roots of aquatic plants like wild rice (approximately a 3 fold induction), as well as in other dryland cereals subjected to oxygen stress (Muench et al. 1993; Good and Crosby 1989). Along with elevated levels of enzyme activity, alanine itself accumulates in plant roots, notably in pea, rice, and barley, under anaerobic conditions (Smith and ap Rees 1979; Hoffman et al. 1986; Reggiani et al. 1988). The concurrent increase in AlaAT activity and alanine production suggest that the elevated levels of alanine biosynthesis are at least partially due to the transamination of pyruvate to alanine by AlaAT, although a correlation also exists between the breakdown of aspartate and an increase in alanine during the early phases of anaerobiosis (Vanlerberghe et al. 1990; 1991; Good and Muench 1993). Production of alanine by AlaAT is thought to play a role in the process of nitrogen assimilation and subsequent protein synthesis within root cells during periods of oxygen stress (Vanlerberghe et al. 1990; 1991; Menegus et al. 1993). Alanine may then be used in the synthesis of aerobic proteins upon the return of oxygenated conditions. Further evidence for the importance of AlaAT and alanine in the anaerobic stress response comes from studies in wild rice, where increases in enzyme activity and levels of alanine occur in both root and leaf tissues (Muench et al. 1993). Taken together, these results support the potential role of AlaAT as an anaerobically important protein and the importance of alanine accumulation in the surviving low oxygen stress.

4.2 Induction of ANPs in *Arabidopsis* by Other Environmental Stresses

Previous studies of the anaerobic stress response in *Arabidopsis thaliana*, along with those described in Chapters 2 and 3, have identified many proteins with potential

roles in the survival of low oxygen conditions imposed by flooding. Further analysis of the expression patterns of these ANPs in *Arabidopsis* indicate that the same proteins may function in this plant's response to a number of different environmental stresses. In addition to hypoxia, the induction of the *Arabidopsis Adh1* gene occurs at the transcriptional level in response to dehydration (10 to 30 fold increase) and cold temperatures (10 to 30 fold at 4 to 5°C) (Dolferus et al. 1994b). Increases in *Adh1* mRNA levels also have been observed with elevated levels of the plant hormone abscisic acid (ABA); rising ABA levels have been associated with dehydration stress (de Bruxelles et al. 1996). Although various sequence motifs of the *Arabidopsis Adh1* promoter are functionally important for transcription under different environmental conditions, a common sequence element was required for *Adh1* induction by cold, dehydration, and hypoxia. Deletion analysis of the *Adh1* promoter delineated a region between bases -172 to -141, upstream of the transcriptional start site, that was critical for gene expression under all three environmental stresses (Dolferus et al. 1994b). This region shares homology with the anaerobic response element found in a number of anaerobically inducible maize genes, including *Adh1* (Walker et al. 1987a,b). However, in *Arabidopsis*, this essential sequence element is important in the plant's responses to a variety of environmental stresses and is referred to as a general stress responsive element (GSRE) (Dolferus et al. 1994a,b).

A similar situation exists for the *AlaAT* gene of *Arabidopsis*. Expression levels of *AlaAT* also have been monitored under environmental stresses other than hypoxia. In the roots of *Arabidopsis*, the *AlaAT* transcript increases by 2 to 3 fold when exposed to conditions of dehydration (Dolferus, unpublished results). Unlike *Adh1*, induction of *AlaAT* expression does not occur during cold or abscisic acid treatments (Dolferus, unpublished results). Based on these preliminary results, *Adh* could have a more general role in the response of *Arabidopsis* to adverse environmental conditions, than *AlaAT*.

4.3 Future Work on the Anaerobic Stress Response in *Arabidopsis*

Despite the identification of an increasing number of anaerobically important proteins, much work remains to be done on the elucidation of the anaerobic stress response in *Arabidopsis*. Specifically in the case of *AlaAT*, the promoter region, along with the 5' end of the gene, have yet to be sequenced. Future studies of the *AlaAT* promoter region will include sequence analysis to determine if the GSRE and related elements are present. The effects of promoter deletions and mutations could be tested using reporter gene constructs to delineate other regions important for the hypoxic induction of *AlaAT* expression. Footprinting analysis would localize sites of protein -

DNA interactions, possibly due to the binding of transcription factors, which may be responsible for the elevated levels of *AlaAT* transcription under hypoxia. These types of studies have already been undertaken with the *Adh1* promoter in *Arabidopsis*. The *Ldh* gene has recently been cloned in *Arabidopsis*, and similar experiments will be performed to obtain a greater understanding of the hypoxic inducibility of *Ldh* (Dolferus, personal communication).

A more general approach for dissecting of the hypoxic signal transduction pathway would involve the generation of mutants affecting various parts of the anaerobic response. Such a mutant screen has been derived for maize seedlings (Bailey-Serres et al. 1988). Varieties of maize, possessing many active transposable elements to increase the frequency of mutation, were germinated underwater. Seeds carrying mutations in anaerobically important genes would likely not germinate under these partially anaerobic conditions, allowing easy separation of anaerobic mutants from wild-type seeds. Once the mutant seeds were removed from the water, germination could occur, and the resulting seedlings could be grown to maturity. Two types of mutants would be isolated from such a mutational screen. Mutations residing in a structural gene encoding an anaerobic polypeptide would affect the functioning of one specific enzyme, whereas a mutation in a regulatory gene, possibly encoding a transcription factor, would limit the activity of several or even all of those ANPs (Bailey-Serres et al. 1988).

A different type of EMS mutagenic screen has been developed in *Arabidopsis*. This mutational scheme involves the use of a variety of reporter gene constructs to select for mutants in the anaerobic signal transduction pathway. The *Adh1* promoter from *Arabidopsis* was attached to a variety of selectable reporter genes, including β-glucuronidase (GUS), hygromycin resistance, and Basta resistance, and these constructs were subsequently transformed into *Arabidopsis* (Good, personal communication). For the *Adh*-GUS transgenics, putative signal transduction mutants would be selected by their inability to activate GUS expression under anaerobic conditions (Good, personal communication). The *Adh*-hygromycin and *Adh*-Basta transgenics would facilitate the selection of mutants in which constitutive gene expression was observed, resulting from the loss of inducibility (Good, personal communication). The isolation of these two types of mutants would help to elucidate the anaerobically important genes involved in the signal transduction pathway governing the anaerobic stress response.

4.4 Concluding Remarks

The results of the experiments described in this thesis indicate that:

1. Hypoxia induces the expression of ADH, LDH, and AlaAT in root tissues of *Arabidopsis thaliana*.

2. The activities of ADH, LDH, and AlaAT may be important in the anaerobic stress response in *Arabidopsis*, although relative levels of metabolites have yet to be measured.

3. A genomic clone corresponding to the *Arabidopsis AlaAT* has been isolated, which will permit further study of the regulation of hypoxic gene expression.

4.5 References

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5 Appendices

5.1 Hoagland's nutrient solution*

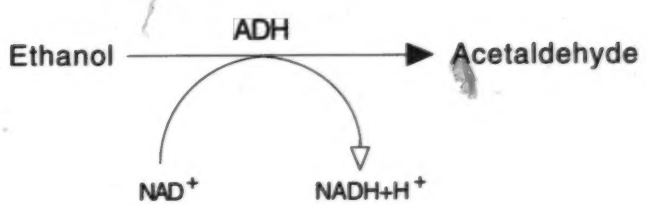
Chemical		20x stock solution (1 liter)[1]	
$Ca(NO_3)_2 \cdot 4H_2O$		18.8 g	(80.0 mM)
$MgSO_4 \cdot 7H_2O$		10.4 g	(42.2 mM)
KNO_3		13.2 g	(0.13 M)
$NH_4H_2PO_4$		2.4 g	(21.0 mM)
FeEDTA		20 ml	
Na_2EDTA	(33.3 g/L)		(2.0 mM)
NaOH	(4.00 g/L)		(2.0 mM)
$FeCl_2 \cdot 4H_2O$	(17.8 g/L)		(2.0 mM)
HCl	(0.15 g/L)		(82.0 µM)
Micronutrients		2 ml	
H_3BO_3	(2.86 g/L)		(93.0 µM)
$MnCl_2 \cdot 4H_2O$	(1.81 g/L)		(18.0 µM)
$ZnSO_4 \cdot 7H_2O$	(0.22 g/L)		(1.5 µM)
$NaMoO_3$	(0.18 g/L)		(1.5 µM)
$CuSO_4 \cdot 5H_2O$	(0.08 g/L)		(0.6 µM)

* The pH of this hydroponic growth solution was adjusted to 6.7 with NaOH.

[1] Half-strength Hoagland's solution was made by adding 500 ml of the stock solution to a hydroponic growth tank containing 20 L of water.

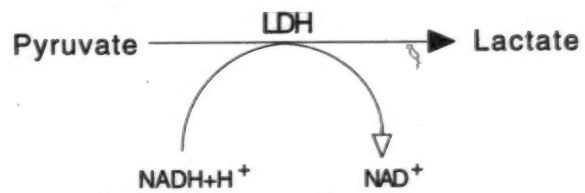
5.2 Enzyme Assay Reactions

5.2.1 Alcohol dehydrogenase



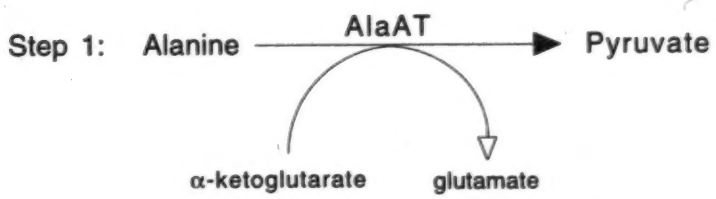
The production of NADH results in an increase of absorbance at 340 nm.

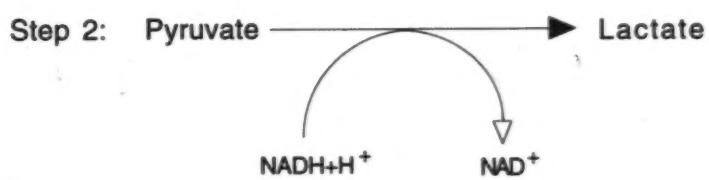
5.2.2 Lactate dehydrogenase



The oxidation of NADH in this reaction causes a decrease in the absorbance at 340 nm.

5.2.3 Alanine aminotransferase





The production of NAD^+ in Step 2 of this assay results in a decrease of absorbance at 340 nm.

5.3 Large scale λ DNA preparation

This protocol was taken from Sambrook et al. (1989) and modified for λ clones carrying AlaAT inserts (pp. 2.67; 2.73-2.77; 2.80-2.81).

5.3.1 Preparation of a primary λ lysate

1) Isolate a single plaque from an agar plate, and place the agar plug into a sterile test tube containing 100 µl of SM buffer. Elute the phage particles from the plug by shaking the tube gently for minimum of 2 h at 4°C.

2) Add 100 µl of a fresh culture of *Escherichia coli LE392*, grown at 37°C for 8 to 12 h (shaking at 150 rpm) in LB media containing 0.2% maltose and 10 mM $MgCl_2$[1]. This mixture of bacteria and phage is incubated at 37°C for 30 min (no agitation)[2].

[1] The bacterial media is supplemented with maltose, as the presence of this sugar induces the maltose operon in *E. coli*, which activates expression of the bacterial *lamB* gene encoding the *lamB* receptor. The λ phage particles adhere to this receptor when infecting *E. coli*. Bacteria grown in the presence of maltose absorb λ phage more efficiently than when this sugar is absent (Sambrook et al. 1989). $MgCl_2$ is included for λ phage particle stability.

[2] This incubation period allows the phage to adhere to the bacteria. The normal incubation time is approximately 20 min (generation time of *E coli*). However, the λ clones containing *Arabidopsis AlaAT* inserts grew better with a slightly longer incubation.

3) Add 1 ml of LB (with 0.2% maltose and 10 mM $MgCl_2$) and incubate at 37°C, with agitation (150 rpm), until complete lysis has occurred (i.e. when the solution becomes completely clear). This takes 5 to 8 h.

4) Add approximately 10 ml of $CHCl_3$ to lyse any remaining bacteria and store at 4°C.

5.3.2 Secondary lysate preparation

1) Mix 1 x 10^8 pfu (plaque forming units) of the primary λ lysate (50 µl to 200 µl) with 5 to 8 ml of *E. coli LE392* (in the exponential growth phase), previously grown in LB media with 0.2% maltose and 10 µM $MgCl_2$. Incubate mixture at 37°C for 30 min (no agitation).

2) Add this mixture to 500 ml of prewarmed (37°C) supplemented LB media in a 2 L flask. Incubate at 37°C with agitation (150 rpm) for 8 to 12 h. The solution may or may not become clear during this time period. Partial lysis is detectable by stringy clumps of bacterial debris in the flask, easily visible upon swirling the solution in the flask.

5.3.3 Extraction of DNA

1) Add 10 ml $CHCl_3$ per 500 ml lysate and shake at 37°C for an additional 15 min to lyse any remaining bacterial cells.

2) Split the lysate into 2-250 ml tubes (leaving $CHCl_3$ behind) and pellet the bacterial debris for 10 min at room temperature (Sorvall centrifuge and GSA rotor used; pelleting speed 10,000 rpm).

3) Transfer the supernatant to new tubes and add DNase and RNase A to a final concentration of 2 µg/ml (50 µl of 10 mg/ml stocks). Incubate at 37°C (no agitation) for 30 min.

4) Add 10 g Na Cl per tube (250 ml lysate) and dissolve. Then incubate the mixture on ice for 1 h. The high salt concentration promotes the dissociation of λ particles from any remaining bacterial debris.

5) Centrifuge for 10 min at 10,000 rpm (4°C).

6) Transfer the supernatant to new GSA tubes, and add 25 g PEG 8000 per tube, dissolve, and incubate at 4°C for 2 h to precipitate the λ phage particles. Centrifuge at 10,000 rpm for 10 min at 4°C. Discard the supernatants.

7) Resuspend each phage pellet in 8 ml of SM buffer. Wash the tube with an extra 2 ml of SM buffer to ensure the complete recovery of phage particles, and pool with the initial 8 ml. Transfer this 10 ml solution to a 30 ml Corex tube.

8) Add 10 ml $CHCl_3$, mix by inversion, and centrifuge at 5,000 rpm for 15 min at 4°C (SA-600 rotor).

9) Transfer the aqueous phase to a SW 28.1 ultracentrifuge tube. Add SM buffer to almost completely fill the tube. (This step prevents tube collapse in the ultracentrifuge.)

10) Spin samples in the ultracentrifuge at 25,000 rpm for 2 h at 4°C in the SW 28.1 rotor to pellet phage particles (extra purification step).

11) Discard supernatant, and resuspend pellet in 1.6 ml of SM buffer. Transfer this solution to a 15 ml Corex tube.

12) Add 2.3% SDS (180 µl of 20% stock) and 11 mM EDTA (35 µl of 0.5 M stock) per tube, and incubate 65°C for 10 min to lyse the phage particles.

13) Add Proteinase K to a final concentration of 0.23 mg/ml (36 µl of 10 mg/L stock) and incubate at 55°C for 1 to 1.5 h to digest the λ proteins.

14) Extract this solution with equal volumes (approximately 2 ml) of phenol, phenol/$CHCl_3$ (1:1 mixture), and $CHCl_3$. For each extraction, mix by inversion, centrifuge at 10,000 rpm (SA-600 rotor) for 15 min at room temperature, to remove contaminating proteins.

15) To the aqueous phase, add 0.1 volume of 3 M NaOAc (pH 7) and 2 volumes of 95% ethanol (ice cold).

16) Pellet the λ DNA at 10,000 rpm for 20 min at 4°C.

17) Wash the pellet with 75% ethanol, and re-pellet as in the step 16.

18) Resuspend the λ DNA in 250 to 500 µl of TE buffer.

19) Measure OD_{260} to determine the DNA concentration and purity (OD_{260}/OD_{280}) of the DNA preparation.

5.4 Large-scale plasmid DNA preparation

This procedure was modified from Sambrook et al. (1989), pp. 1.38-1.41.

1) Inoculate 250 ml of LB Amp (LB media with 50 µg/ml ampicillin) with a single transformed bacterial colony (*E. coli DH5α* strain used). Incubate at 37°C with agitation (150 rpm) for 12 to 16 h.

2) Transfer culture to GSA centrifuge tubes and spin at 5,500 rpm for 15 min at 4°C.

3) Discard the supernatant and resuspend the bacterial pellet in 6 ml of freshly prepared lysis buffer (25 mM Tris [pH 8], 10 mM EDTA [pH 8], 15% sucrose, 2 mg/ml lysozyme). Incubate on ice for 20 min.

4) Add 12 ml of freshly prepared 0.2 M NaOH, 1% SDS buffer, and carefully mix by inversion. Incubate on ice for 20 min.

5) Add 7.5 ml of 3 M NaOAc (pH 4.6), and mix gently by inversion. Incubate on ice for 20 min. The low pH of the NaOAc facilitates the preferential precipitation of the bacterial DNA (high molecular weight), while the plasmid DNA remains in solution.

6) Transfer the solution to Oakridge tubes, and pellet the bacterial debris and DNA at 15,500 rpm for 15 min at 4°C (SA-600 rotor used).

7) Transfer the supernatant to new Oakridge tubes, leaving the pelleted debris behind. An additional 10 min centrifugation was performed to ensure the complete removal of bacterial debris.

8) Add RNase A to a final concentration of approximately 15 µg/ml (50 µl of 10 mg/ml stock) per tube, and incubate at 37°C for 20 min.

9) Add 4 ml each of phenol and $CHCl_3$ per tube, mix well, and centrifuge at 8,500 rpm for 15 min at room temperature, to remove contaminating proteins.

10) Transfer the aqueous layer to a new tube, add 2 volumes of 95% ethanol (ice cold) and incubate on ice for 30 min to precipitate the DNA.

11) Pellet the DNA at 8,000 rpm for 20 min at 4°C.

12) Briefly dry the pellet (2 min in 65°C oven), and redissolve the pellet in 1.6 ml of water. Then add 1 M NaCl and 6% PEG (final concentrations), and incubate at 4°C for 1 h to overnight. In this step, preferential precipitation of plasmid DNA occurs, leaving any contaminating bacterial DNA (not previously removed) in solution.

13) Pellet the plasmid DNA by centrifugation at 10,500 rpm for 20 min at 4°C. Discard the supernatant, and wash the pellet with 5 ml of 75% ethanol. Re-centrifuge and discard the supernatant.

14) Allow the pellet to dry (in air or 65°C oven), and resuspend in 1 ml of TE buffer. Measure the OD_{260} to determine the DNA concentration.

5.5 Plant DNA extraction

This protocol was modified from Doyle and Doyle (1990).

1) Grind 2 g of leaf tissue in N_2 (l) using a mortar and pestle. Transfer the powder to an Oakridge tube.

2) Add 15 ml 2x CTAB buffer, prewarmed to 60°C, after all N_2 (l) has evaporated. Incubate the mixture for 30 min in a 60°C water bath.

3) Add 15 ml $CHCl_3$ mix by inversion, and centrifuge at 15,000 rpm for 10 min at room temperature. Repeat the $CHCl_3$ step if the supernatant is still a dark green color.

4) Transfer the aqueous phase to a new tube, and add 0.6 volumes of isopropanol to precipitate the DNA and RNA.

5) Centrifuge at 5,000 rpm for 5 min at room temperature to pellet the nucleic acids. Discard the supernatant, and wash the pellet with 70% ethanol for 20 min to overnight. Re-pellet the DNA, and discard the ethanol.

6) Dissolve the pellet in 1 ml TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]), and add 0.1 mg/ml RNase A (final concentration) to remove contaminating RNA molecules. Incubate at 37°C for 30 min.

7) Add 2 ml of water, along with 2.5 M NH_4 OAc (final concentration) and 0.6 volumes of isopropanol. Centrifuge at 5,000 rpm for 5 min at room temperature.

8) Discard the supernatant, and wash the pellet in 10 ml of 70% ethanol for 20 min at room temperature. Re-pellet DNA as in step 7.

9) Remove the supernatant, and dissolve the DNA pellet in 1 ml of TE (pH 8.0).

10) DNA concentrations can be estimated using gel electrophoresis, as any residual CTAB will interfere with spectrophotometer readings.

2X CTAB buffer

1.4 M NaCl

20 mM EDTA (pH 8.0)

100 mM Tris (pH 8.0)

2% (w/v) CTAB

0.2% (v/v) 2-mercaptoethanol (added fresh)

CTAB = hexadecyltrimethylammonium bromide (purchased from Sigma)

5.6 Capillary blotting

This procedure was outlined by Southern (1975) and modified from the GeneScreen Plus protocol booklet (New Research Products).

1) If transferring large fragments of DNA, submerse agarose gel in a 0.25 M HCl solution for 15 min with gentle agitation. The acid-nicking of high molecular weight DNA molecules facilitates their transfer. If the bromophenol blue dye front is still on the gel, it should turn to a light yellow color.

2) Drain off the HCl solution, and replace it with a denaturing solution (0.5 M NaOH, 1.5 m NaCl). Slowly agitate the gel in denaturing solution for 30 min, then drain off the solution and add fresh denaturing solution. Leave the gel shaking for another 15 min. (The bromophenol blue dye should return to its blue color.)

3) Replace the denaturing solution with a neutralizing buffer (0.5 M Tris [pH 7.5], 1.5 M NaCl) for 30 min. Drain off the solution, and add fresh neutralizing buffer for 15 min more.

4) While the gel is neutralizing, cut a piece of GeneScreen Plus membrane to the exact size of the gel, handling only the edges of the membrane (while wearing gloves).

5) Lay the membrane on the surface of distilled water (in a container) such that one side wets, then shake to wet the other. When thoroughly wetted, replace the water with 10X SSPE and let the membrane soak for 15 min.

6) Pour 10X SSPE into a glass container. Cut 2 pieces of Whatman 3 MM paper (same width as the gel but considerably longer), and wet them in 10X SSPE. Form a wick by wrapping the 3 MM paper over a glass plate (larger than the gel) supported on a petri dish in the glass container. Smooth all the bubbles and wrinkles out of the wick using a test tube as a roller. In addition to this setup, have 3 more pieces of 3 MM paper (gel size), a stack of paper towels (gel size) a second glass plate (larger than the gel), and a weight (i.e. a 50 ml bottle filled with water).

7) When neutralization is complete, lift the gel from the solution and place it wells-side down on the wick. Gently smooth out any bubbles between the wick and the gel.

8) Place the membrane on the gel. Use one smooth motion, letting the center touch first, then moving towards the sides of the gel. Gently remove any bubbles trapped between the gel and the membrane.

9) Place the 3 dry pieces of 3 MM paper on the membrane, followed by the paper towels, the second glass plate, and finally the weight.

10) Transfer for approximately 16 to 20 h.

11) Carefully remove the paper towels and 3 MM paper. While wearing gloves, peal off the membrane. If the transfer has worked, the gel will be thin and tough. The gel can be rehydrated and stained to determine the efficiency of DNA transfer.

12) Place the membrane in 0.4 M NaOH for 1 min (shaking) to ensure that all the transferred DNA has been denatured.

13) Rinse the blot in 2X SSPE, then agitate the membrane in this solution for 5 min.

14) Store the filter sandwiched between 2 sheets of 3 MM paper. The blot must be completely dry before continuing (air dry at least 30 min).

5.7 References

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